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(54) Title: CHIMAERIC PLANT VIRUSES WITH MUCIN PEPTIDES

(a)

Sequence of SBMV Coat Protein Spanning The Potential Insertion Site With Introduced Base
Changes and New Restriction Sites: (sequence starts at nt 3955)

```
M E G G S S K T A V N T G
ATGGAAGGAGGATCATCTAAGACTGCTGTGAACACTGGG
      ↓           ↓
    GGATCC      GTTAAC
      BamHI      HpaI
```

(b)

Series of Sequences to be Inserted Between the Restriction Sites to Insert the MUC1(16)
Epitope at Various Locations

```
G V T S A P D T R P A P G S T A
GGTGTTACTTCTGCTCCTGATACTAGACCTGCTCCTGGTTCTACTGCT
CCACAATGAAGACGACCACTATGATCTGGACGAGGACCAAGATGACGA
```

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      ↓           ↓
GATCC   TCTAAGACTGCTGTT
  G      AGATTCTGACGACAA

GATCCTCT AAGACTGCTGTT
  GAGA    TTCTGACGACAA

GATCCTCTAAG ACTGCTGTT
  GAGATTC    TGACGACAA

GATCCTCTAAGACT GCTGTT
  GAGATTCTGA    CGACAA

GATCCTCTAAGACTGCT GTT
  GAGATTCTGACGA    CAA
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(57) Abstract: Mucin peptide epitopes are inserted into
the coat protein of a plant virus (e.g. a comovirus such as
CPMV) having a beta-barrel structure at an immunogeni-
cally effective site, such as in a loop connecting beta sheets
or at/near the C-terminus. The resulting chimaeric virus
particles are extremely immunogenic, giving better results
than KLH conjugation and not requiring the addition
of exogenous adjuvant. They are effective at mucosal
surfaces, particularly when administered intranasally.



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CHIMAERIC PLANT VIRUSES WITH MUCIN PEPTIDES

All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

5 This invention is in the field of vaccination, more particularly cancer vaccines. It relates to antigenic materials peptides which are rendered more antigenic by being presented on carrier virus particles. The peptides are able to induce antibodies to tumour antigens.

BACKGROUND ART

10 Based on the success of vaccines for the treatment and prevention of infectious diseases, there have been attempts over the years to develop cancer vaccines. Early attempts at cancer vaccines were based on immunising the cancer patient with killed tumour cells together with adjuvant.

Further development of cancer vaccines has focused on directing the immune response to identified tumour antigens present on the surface of tumour cells. The advent of monoclonal antibodies enabled the identification of so-called "tumour-specific" antigens. Typically, however, tumour antigens not tumour-specific, but are over-expressed on tumour cells and
15 rarely expressed, or under-expressed, on normal cells.

A well-characterised tumour antigen is "polymorphic epithelial cell mucin" (PEM), the product of the MUC1 gene. PEM is over-expressed and aberrantly glycosylated in many carcinomas (breast, pancreas, ovary, lung, urinary bladder, prostate, and endometrium) thus resulting in cancer cells expressing antigenically-distinct PEM molecules on their surface. Both humoral
20 and cellular immune responses specific to PEM have been detected in cancer patients. These properties make PEM particularly well-suited to act as a target molecule for cancer immunotherapy. In addition, the over-expression of mucin on tumour cells suggests that the mucin may be important for the maintenance of the tumour. Such over-expression of mucin would alleviate potential problems of tumour cells escaping immunotherapy by down-
25 regulating the MUC1 gene. US patents 4,963,484 & US 5,053,489 and international patent application WO88/05054 describe the purification of human mucin, the isolation of monoclonal antibodies thereto, the cloning of cDNAs coding for human mucin, and the use of these for diagnostic and therapeutic applications.

30 Zhang *et al.* [*Cancer. Res.* (1996) 56:3315-3319] disclose the preparation of several MUC1 peptide vaccines and compares their immunogenicity. Vaccines comprising adjuvant and a

MUC1 peptide covalently linked to the protein carrier KLH achieved greatest immunogenicity. The authors concluded that MUC 1 peptides of 30 or more amino acids are better immunogens than MUC 1 peptides of 20 amino acids.

5 The MUC1 gene product is a high molecular weight (MW>200kDa) transmembrane glycoprotein expressed on the apical surface of many simple epithelial cells. It has a relatively large extracellular domain varying from 1000 to 2200 amino acids and a cytoplasmic tail of 69 amino acids. The extracellular domain consists mainly of tandem repeats of the 20 amino acid sequence PDTRPAPGSTAPPAHGV TSA [for a review, see Apostolopoulos & McKenzie (1994) *Crit. Rev. Immunol.*, 14: 293-309]. Variability in the number of repeats accounts for the
10 variability in the size of the extracellular domain. Each repeat contains five potential O-linked glycosylation sites and two or possibly three of these sites are believed to be utilised.

The aberrant glycosylation of PEM observed in a variety of cancers is due to alterations in the activity of glycosyltransferases with some transferases being inactive and the activity of others being increased. Abnormal glycosylation of PEM in cancer cells leads to three types of cancer-
15 associated epitopes: (a) peptides, from the core protein, that are exposed following under-glycosylation; (b) "new" carbohydrates resulting from deficient and aberrant glycosylation; and (c) "new" glycopeptides resulting from deficient and aberrant glycosylation.

Many different approaches have been considered as potential cancer vaccines aimed at inducing a beneficial immune response directed towards PEM-expressing tumour cells. These include:
20 cells expressing mucins; mucin purified from cancer cells; mucin core protein produced as a recombinant protein; different mucin glycoproteins produced by transfected cells; peptide or glycopeptide based on the tandem repeat; recombinant animal viruses expressing portions of the MUC1 cDNA gene product; synthetic carbohydrates based on short aberrant chains present on mucins on cancer cells; naked MUC1 DNA; and anti-MUC1 antibodies.

25 Of these, anti-MUC1 antibodies, synthetic carbohydrates, recombinant animal viruses, and peptides based on the tandem repeat have been developed sufficiently to reach evaluation in clinical trials. However, one significant problem with all of the above approaches is that a poor and/or inadequate immune response towards mucin-expressing tumour cells is observed.

It is an object of the present invention to alleviate this problem by providing an effective means
30 for presenting a peptide of a tumour-associated mucin.

DISCLOSURE OF THE INVENTION

According to a first aspect of the invention, there is provided a chimaeric virus particle (CVP) derived from a plant virus, having a coat protein with a beta-barrel structure, and modified by insertion of an immunogenically active peptide of a tumour-associated mucin at an immunogenically effective site in the coat protein.

These CVPs provide, *inter alia*, two main advantages over the above-identified prior art antigen-presenting means. Firstly, conventional live animal virus vectors (as carriers) can be dispensed with. Secondly, the need for separate mucin peptide synthesis and the need for time-consuming chemical-coupling thereof to a conventional carrier, such as keyhole limpet haemocyanin (KLH), can be avoided. In addition, the CVPs have been shown to induce good mucosal immunity (*e.g.* when administered intranasally), they are superior to the 'standard' of presentation by conjugation to KLH [Zhang *et al.*, *supra*], they do not require the addition of exogenous adjuvants to induce a strong immune response, and show good Th1 bias.

It has been found that surprisingly superior immunogenicity to a mucin antigen can be obtained by presenting a mucin peptide in a composite particle derived from certain plant viruses. The plant viruses especially useful for the purposes of the present invention are those having a coat protein with a β -barrel structure containing loops between β -sheets. Composite virus particles of this general type and the methods of constructing such particles are disclosed in international patent applications WO92/18618 and WO96/02649.

An advantage of the use of viruses which have a β -barrel structure is that the loops between the individual strands of β -sheet provide convenient sites for the insertion of mucin peptides. Modification of one or more loops is a preferred strategy for the expression of mucin peptides in accordance with the present invention. These viruses include all members of the following virus families: *Caulimoviridae*, *Bromoviridae*, *Comoviridae*, *Geminiviridae*, *Reoviridae*, *Partitiviridae*, *Sequiviridae*, *Tombusviridae*, and the following virus genera: Luteovirus, Marafivirus, Sobemovirus, Tymovirus, Enamovirus and Idaeovirus. Of the *Tombusviridae* family, the following genera are mentioned in particular: Dianthovirus, Machlomovirus and Necrovirus. An advantage of the *Comoviridae* and *Sequiviridae* is that their capsid contains sixty copies each of 3 different β -barrels which can be individually manipulated. All other virus families and genera listed above have similar 3-dimensional structures but with a single type of β -barrel. Viruses selected from the family *Comoviridae* (*e.g.* cowpea mosaic virus and bean pod mottle virus) are particularly preferred, with CPMV being the most preferred virus.

The invention can be applied to any plant virus having a coat protein with a β -barrel structure. In a preferred embodiment the three dimensional structure of a plant virus is examined in order to identify portions of a coat protein which are particularly exposed on the virus surface and which are therefore potentially optimum sites for insertion. In a further embodiment the amino acid sequence of the exposed portions of a coat protein is examined for amino acids which break α -helical structures because these are potentially optimum sites for insertion. Examples of suitable amino acids are proline and hydroxyproline, both of which whenever they occur in a polypeptide chain interrupt the α -helix and create a rigid kink or bend in the structure.

All plant viruses possessing icosahedral symmetry whose structures have been solved conform to the eight stranded β -barrel fold as exemplified by CPMV, and it is likely that this represents a common structure in all icosahedral viruses. All such viruses are suitable for use in this invention for the presentation of foreign peptide sequences in the loops between the β -strands.

To date, viruses from nine plant virus genera and three subgroup 2 ssRNA satellite viruses have had their tertiary and quaternary structures solved at high resolution. These are:

15	Name	Acronym	Genus	Family
	Southern bean mosaic virus	SBMV	<i>Sobemovirus</i>	not assigned
	Sesbania mosaic virus	SMV	<i>Sobemovirus</i>	not assigned
	tomato bushy stunt virus	TBSV	<i>Tombusvirus</i>	<i>Tombusviridae</i>
	turnip crinkle virus	TCV	<i>Carmovirus</i>	<i>Tombusviridae</i>
20	cowpea chlorotic mottle virus	CCMV	<i>Bromovirus</i>	<i>Bromoviridae</i>
	alfalfa mosaic virus	AMV	<i>Alfamovirus</i>	<i>Bromoviridae</i>
	bean pod mottle virus	BPMV	<i>Comovirus</i>	<i>Comoviridae</i>
	cowpea mosaic virus	CPMV	<i>Comovirus</i>	<i>Comoviridae</i>
	red clover mottle virus	RCMV	<i>Comovirus</i>	<i>Comoviridae</i>
25	tobacco ringspot virus	TRSV	<i>Nepovirus</i>	<i>Comoviridae</i>
	turnip yellow mosaic	TYMV	<i>Tymovirus</i>	not assigned
	tobacco necrosis virus	TNV	<i>Necrovirus</i>	<i>Tombusviridae</i>
	satellite tobacco necrosis virus		<i>Subgroup 2</i>	
	satellite panicum mosaic virus		<i>Subgroup 2</i>	
30	satellite tobacco mosaic virus		<i>Subgroup 2</i>	

The similarity of the secondary structural elements and their spatial organisation is illustrated in Figure 1. Any of the loops which lie between the β -strands can be used for insertion of foreign epitopes, but the insertions are made such that the additions are exposed on either the internal or external surface of the virus and such that assembly of the coat protein subunits and the infectivity of the virus are not abolished. The choice of a particular loop can be made using knowledge of the structure of individual coat protein subunits and their interactions with each other, as indicated by the crystal structure, such that any insertions are unlikely to interfere with virus assembly. The choice of precise insertion site can be made, initially, by inspection of the crystal structure, followed by *in vivo* experimentation to identify the optimum site.

- 10 The present invention is also applicable to those β -barrel containing plant viruses whose crystal structures have not yet been determined. Where significant sequence homology within the coat protein genes exists between one virus whose crystal structure is unknown and a second virus whose crystal structure has been determined, alignment of the primary structures will allow the locations of the loops between the β -strands to be inferred [Dolja & Koonin (1991) *J. Gen. Virol.* 72:1481-1486]. In addition, where a virus has only minimal coat protein sequence homology to those viruses whose crystal structure has been determined, primary structural alignments may be used in conjunction with appropriate secondary and tertiary structural prediction algorithms to allow determination of the location of potential insertion sites.

CPMV comprises two subunits, the small (S) and the large (L) coat proteins, of which there are 60 copies of each per virus particle. Mucin peptide sequences may be expressed on either the L or S proteins or on both coat proteins on the same virion. Thus, up to 120 copies of the mucin peptide sequence may be expressed on a single virus particle.

A 3.5Å electron density map of CPMV (see Figure 1 in WO92/18618) shows the clear structural relationship between the capsids of CPMV and the T=3 plant viruses, for example the bromoviruses (in particular CCMV) and the sobemoviruses (in particular SBMV). The capsids of these latter viruses are composed of 180 identical coat protein subunits, each consisting of a single β -barrel domain. These domains can occupy three different positions, namely A, B and C, within the virions (see Figure 1 in WO92/18618). The two coat proteins of CPMV were shown to consist of three distinct β -barrel domains, two being derived from the large capsid protein and one from the small capsid protein. Thus, in common with the T=3 viruses, each CPMV particle is made up of 180 β -barrel structures. The single domain from the small subunit occupies a position analogous to that of the A type subunits of CCMV and SBMV and other

viruses, whereas the N- and C-terminal domains of the large capsid protein occupy the positions of the C and B type subunits respectively (see Figure 1 in WO92/18618).

X-ray diffraction analysis of crystals of CPMV and BPMV shows that their 3-D structures are very similar and are typical of the *Comoviridae* in general.

- 5 In the structures of CPMV and BPMV, each β -barrel consists principally of 8 strands of antiparallel β -sheet connected by loops of varying length. The connectivity and nomenclature of the strands is given in Figure 2 of WO92/18618. The flat β -sheets are named the B,C,D,E,F,G,H and I sheets, and the connecting loops are referred to as the β B- β C, β D- β E, β F- β G and β H- β I loops.
- 10 One difference between the *Comoviridae* and the animal *Picornaviridae* is that the protein subunits of *Comoviridae* lack the large insertions between the strands of the β -barrels found in *Picornaviridae*. The four loops (β B- β C, β D- β E, β F- β G and β H- β I – see Figure 3 in WO92/18618) between the β -sheets are suitable for expression of tumour-associated mucin peptide sequences.
- 15 The β B- β C loop in the small capsid protein is particularly preferred as the insertion site. This loop has an engineered *Aat*II site and a unique *Nhe*I site at position 2708 of the M RNA-specific sequence where mucin peptide sequences may be inserted (see Figure 4 of WO92/18618). The insertion site immediately preceding Pro²³ in the β B- β C loop of the small capsid protein is most preferred.
- 20 To demonstrate the present invention, the plant virus CPMV in particular has been primarily chosen.

Various sites in the CPMV coat protein have been identified as suitable for insertion of the foreign peptide. The co-ordinates given below refer to the linear amino acid sequence of the CPMV coat protein (S or L subunit).

- 25 Any insertion site which does not lie between the N-terminus of a subunit and a β -strand, or between a β -strand and the C-terminus, is considered to lie between two β -strands. Such an insertion site may lie in a short loop at one of the axes of symmetry of the virus or in one of the much longer connecting strands which form the body of the protein subunits and which may contain additional secondary structure and form loops on the surface of the virus. In particular,
30 there are α -helices present in some of the connecting strands which form the body of the protein

subunits, and the co-ordinates given for some of the insertion sites may indicate that an α -helix is present between the insertion site and the preceding or proceeding β -strand. For example, the S protein C' and C'' β -strands represent a secondary structure formed in the loop between the β C and β D strands.

5 (i) External Surface Sites

S Subunit (A Domain) Insertion Sites

β B- β C: The residue between the β -strands are Thr 19 to Val 2, and the preferred insertion site is between amino-acids 22 and 23. Insertion sites either side of this are also suitable, notably between residues 21 and 22.

10 β C'- β C'': The residues between the β -strands are Val 42 to Asn 46.

β H- β I: This site is at the tip of the five-fold axis and the residues between the β -strands are Thr 152 to Gln 158.

β D- β E: Again, this site is at the tip of the five-fold axis and the residues between the β -strands are Ala 80 to Gln 90.

15 β E- β F: This site is not at the tip of the five-fold axis, but lies 'behind' and to one side of the β -strands. The residues between the β -strands are Arg 96 to Ala 106. Residues 98 to 102 are preferred.

L Subunit (B Domain) Insertion Sites

20 The B domain of the L subunit comprises amino acids 183-374 of the linear amino-acid sequence.

β B- β C: This site is in the equivalent location on the subunit to the standard S protein insertion site and is at the three-fold axis of the virus. The residues between the β -strands are Pro201 to Glu209.

25 β H- β I: Again this site is at the three-fold axis of the virus and the residues between the β -strands are His331-Asp341.

β C- α A (β C- β D): This site lies between the β C and β D strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α -helix (termed α A)

and the insertion site is a surface exposed portion which lies between the β C strand and the α A helix. The surface exposed residues are Ala 223 to Ala226.

5 β G- α D (β G- β H): This site lies between the β G and β H strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α -helix (termed α D) and the insertion site is a surface portion which lies between the α D helix and β H strands which are surface exposed. The surface exposed residues are Pro314 to Thr317.

10 β E- α B (β E- β F): This site lies between the β E and β F strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α -helix (termed α β) and the insertion site is a surface portion which lies between the β E strand and the α B helix. The surface exposed residues are Gly269 to Phe275.

L subunit (C Domain) Insertion Sites

The C Domain of the L Subunit comprises amino-acids 1-182 of the linear amino-acid sequence.

15 β E- α B (β E- β F): This site lies between the β E and β F strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α -helix (termed α B) and the insertion site is a surface exposed portion which lies between the β E strand and the α B helix. The surface exposed residues are Gly95 to Thr102.

20 α D- β H (β G- β H): This site lies between the β G and β H strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α -helix (termed α D) and the insertion site is a surface portion which lies between the α D helix and the β H strands. The surface exposed residues are Ser142 and Arg145.

25 β - α A (β C- β D): This site lies between the β C and β D strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α -helix (termed α A) and the insertion site is a surface exposed portion which lies between the β C strand and the α A helix. The surface exposed residues, not part of any secondary structural element, are Gly53 to Phe56.

β B- β C: This site is an equivalent location on this domain to the S protein β B- β C (identified above) insertion site and is at the three-fold axis of the virus. The residues between the β -strands are Ser33 to Leu42.

(ii) Internal Surface Sites**S Subunit (A Domain) Insertion Sites**

β -G- β H: This protein chain between β -strands points in towards the interior of the virus and forms a 'double loop'. One insertion site comprises residues Pro128 to Ser130.

5 L Subunit (B Domain) Insertion Sites

β F- β G: This loop is at the three-fold axis symmetry of the virus, and is the bottom loop of the four. The residues in the loop are Gln287 to Glu293.

C Domain β I-B Domain β B: This is the junction between the B and C domains of the L subunit. This linker sequence comprises residues Asn374 to Asp186. The insertion site is
10 around Ala185, which is assigned to the B domain.

L Subunit (C Domain) Insertion Sites

β G- α D (β G- β H): This site lies between the β G and β H strands. The protein chain loops out to form part of the body of the protein domain. Within this loop is an α -helix (termed α D) and the insertion site is an internal projecting loop which lies between the β G strands and
15 the α D helix. The residues in this loop are Asn130 to Ser135.

The RNA or DNA encoding the mucin peptide may be inserted into the plant virus genome in a variety of configurations. For example, it may be inserted as an addition to the existing nucleic acid or as a substitution for part of the existing sequence, the choice being determined largely by the structure of the capsid protein and the ease with which additions or replacements can be
20 made without interference with the capacity of the genetically-modified virus to assemble. Determination of the permissible and most appropriate size of addition or deletion for the purposes of this invention may be achieved in each particular case by experiment in the light of the present disclosure. The use of addition inserts appears to offer more flexibility than replacement inserts in some instances.

25 The mucin peptides which may be incorporated into plant viruses according to this invention may be of highly diverse types and are subject only to the limitation that the nature and size of the peptide and the site at which it is placed on or in the virus particle do not interfere with the capacity of the modified virus to assemble when cultured *in vitro* or *in vivo*. The peptide preferably contains 5 or more amino acids.

The following are preferred mucin peptides sequences for forming CVPs in accordance with the present invention. Optionally repeating sequences of the 20 amino acid sequence "PDTRPAPGSTAPPAHGV TSA" (SEQ. ID No.1) are preferred. An optionally repeating partial sequence of the above 20 amino acid sequence is particularly preferred. In this respect the peptide sequences "PDTRP" (SEQ. ID No.2) and "APDTR" (SEQ. ID No.3) are particularly preferred, as are the mimetic peptide sequences: "DAHWESWL" (SEQ. ID No.4) and "DLHWASWV" (SEQ. ID No.5).

The mucin peptide may be of the general formula "(aa)_xPDTRP(aa)_y", or "(aa)_xAPDTR(aa)_y" where aa is an amino acid residue, the same or different in each position, x is an integer from 0 to 1000, and y is an integer from 0 to 1000. Preferably x is an integer from 0 to 500, more preferably 0 to 100, most preferably 0 to 10, and y is an integer from 0 to 500, more preferably 0 to 100, most preferably 0 to 10.

It is most preferred that the sequence "PDTRP" or "APDTR" is located towards the middle of the mucin peptide sequence of interest (as for example in the MUC1(16) defined hereinafter). Alternatively, the peptide sequence "PDTRP" or "APDTR" may be located towards the beginning of the mucin peptide sequence of interest (as for example in the MUC1(23) defined hereinafter). The above sequences/partial sequences are based on the 20 amino acid tandem repeat sequence of the extracellular of PEM.

According to a second aspect of the invention, there is provided a method of producing a chimaeric virus particle which comprises introducing a nucleotide sequence coding for a tumour-associated mucin peptide to modify the plant viral nucleic acid which codes for the coat protein, infecting plants, plant tissue, plant cells, or protoplasts with the modified viral nucleic acid, and harvesting chimaeric virus particles. The introduced nucleotide sequence is preferably inserted in that part of the plant viral nucleic acid which codes for an exposed region of the coat protein.

This procedure is best carried out by direct manipulation of the DNA of the virus in the case of DNA viruses or by manipulation of the cDNA corresponding to the RNA of an RNA virus. In the case of an RNA virus, the modified cDNA or an RNA transcript thereof is prepared for inoculation of plant cells or preferably whole plants so as to achieve a multiplication stage prior to the harvesting of assembled particles of the modified virus. In the case of a DNA virus, the DNA itself is introduced into the plant. In this way, the mucin peptide is initially expressed as part of the capsid protein and is thereby produced as part of the whole virus particle.

In order to produce modified virus on a commercial scale, it is not necessary to prepare infective inoculant (DNA or RNA transcript) for each batch of virus production. Instead, an initial inoculant may be used to infect plants and the resulting modified virus may be passaged in the plants to produce whole virus or viral RNA as inoculant for subsequent batches.

- 5 Preferably the method is applied to an RNA plant virus, in which case the method comprises introducing a DNA coding for the tumour-associated mucin peptide into a cDNA corresponding to the RNA of the plant virus which codes for an exposed portion of its coat protein, inoculating plants, plant tissue, plant cells, or protoplasts with the thus modified cDNA or an RNA transcript thereof, if necessary together with any other DNA or RNA required for multiplication
10 and assembly of whole virus particles in the plant material, and harvesting chimaeric virus particles. More preferably, the modified cDNA is produced by introducing the DNA encoding the mucin peptide into a DNA fragment excised from the plant viral cDNA, and reinserting the modified excised fragment so as to constitute the plant viral cDNA in modified form.

- According to a third aspect of the invention, there is provided a vaccine comprising CVPs, as
15 hereinbefore described, as an immunogenic component thereof. The vaccine may further comprise adjuvant, for example Freund's complete adjuvant (FCA), QuilA, QS-21, ISCOM matrix, algamulin, alum, or combinations thereof. QS-21 is preferred. Alternatively, adjuvant may be omitted from the vaccine. It is particularly surprising that the chimaeric virus particles of the present invention are strongly immunogenic in the absence of an adjuvant (*e.g.* see
20 Examples 7 & 8).

- Vaccines according to the present invention are particularly suited for nasal administration. For comparison purposes, a chimaeric plant virus particle comprising alfalfa mosaic virus coat protein and a rabies virus peptide is described in Modelska *et al.* [*PNAS USA* (1998) 95:2481-85]. The chimeric plant virus coat protein assembled *in planta* into virus-like particles which (in
25 contrast to the CVPs of the present invention) were non-infectious to plants. Three doses of 50µg of the virus-like particles were required to obtain an immune response when administered to mice by injection in the absence of an adjuvant. In contrast, a strong immune response was achieved in accordance with the present invention by either subcutaneous or nasal administration to mice of two 100µg doses of CPMV-MUC1(16) in the absence of an adjuvant
30 (see Examples 7 & Example 8). The nasal vaccines of the invention may be adapted for intranasal administration, such as by nasal spray, nasal drops, gel or powder [Almeida & Alpar (1996) *J. Drug Targeting* 3:455-467].

Similarly high post-immunisation (day 28) titres were seen in mice immunised with either 0.5µg or 5µg CPMV-MUC1(16) on days 0 and 21. The 0.5µg dose gave a titre comparable to the previous 50µg KLH-MUC1 experiment.

Example 5 – CPMV is a more effective MUC1 carrier than KLH in MUC1-transgenic mice

- 5 To be effective in humans, a MUC1-based vaccine must be able to overcome immune privilege and elicit MUC1-specific antibodies when present as a self antigen. In the previous examples, the MUC1 peptides were not functioning as self antigens, but were foreign (mouse and human sequences ~30% homology). To address this issue, mice transgenic for human MUC1 were tested [Graham, *et al.* (1996) *Int. J. Cancer* 65:664-670] as a better model of humans.
- 10 Transgenic mice expressing human MUC1 and non-transgenic mice were immunised subcutaneously with 100µg of CPMV-MUC1(16), wild type CPMV or 25µg MUC1-KLH, using QuilA adjuvant, and boosted with the same dose on day 14. Sera were pooled for mice in the same group, except for the transgenic mice immunised with CPMV-MUC1(16) which were analysed individually (Fig. 5).
- 15 CPMV-MUC1(16) was shown to be highly immunogenic in human MUC1-transgenic mice with anti-MUC1 peptide antibody titres only a little lower than those elicited in non-transgenic mice (Fig. 5). Moreover, the anti-MUC1 peptide antibodies elicited in the transgenic mice by CPMV-MUC1(16) immunisation were considerably greater than those elicited by the MUC1 peptide conjugated to KLH (Fig. 5). The average titres of anti-MUC1 antibodies were 40,500
- 20 following immunisation with CPMV-MUC1(16), 400 following immunisation with MUC1-KLH, and 120 following immunisation with wild type CPMV (see Fig. 5). These data confirm and further support the enhanced presentation system of the present invention by demonstrating that the presentation of a mucin peptide on the surface of a plant virus is significantly better than the presentation as conjugated to KLH (the conventional carrier).
- 25 In summary, over 10-fold less peptide was presented on CPMV as compared to KLH, and a 100-fold higher level of anti-peptide antibody was induced by CPMV-MUC1(16) as compared to corresponding presentation by KLH (1,000-fold difference in total).

Example 6 – relative efficacy of adjuvants to augment CPMV-MUC1(16) responses

- Because FCA is not a suitable adjuvant for human use, further studies were conducted with
- 30 CPMV-MUC1(16) adjuvanted with less toxic adjuvants including the saponin-based adjuvants QuilA (20µg/dose), QS-21 (20µg/dose) and ISCOM matrix (10µg/dose), or with algamulin (a

mixture of alum and gamma-inulin; 10µg/dose). Mice were immunised subcutaneously with 50µg CPMV-MUC1(16) in the above adjuvants and boosted with 25µg on days 14 and 28 with the same adjuvant.

Mice immunised with CPMV-MUC1(16) in QS-21 elicited higher titres of MUC1-specific antibody than all other adjuvants tested (Fig. 6), including FCA, when assayed on day 42. Titres in all the adjuvant groups remained high for 70 days and then began to drop, except for the FCA and QS-21 groups which still had high titres on day 150 (mean titres of 76,800 and 57,600 respectively; Fig. 6).

Thus QS-21 facilitates optimal and sustained responses to CVPs and, together with its favourable safety profile, provides a suitable choice as an adjuvant for inclusion in a CVP-based cancer vaccine for human use.

Example 7 – exogenous adjuvant is not required for CVPs of the present invention

Mice were immunised subcutaneously with 100µg of CPMV-MUC1(16) on days 0 and 21 with QS-21 and on days 0 and 14 without QS-21 as adjuvant. On day 35, the titres of the anti-MUC1 peptide antibodies were not very different between the mice immunised with or without adjuvant (see Fig. 4). Although the use of QS-21 is beneficial in increasing, and probably also prolonging, the immune response to the mucin peptide present on the CPMV particle, the use of an adjuvant such as QS-21 is not essential. CPMV-MUC1(16) administered without any adjuvant induced a good immune response to the MUC1 peptide.

Example 8 – intranasal vaccination

Mice were immunised intranasally on days 0, 7, 14, and 21 with 100µg per dose of CPMV-MUC1(16) or wild-type CPMV. The immunisations were performed with or without the use of cholera toxin (CT). CT has nasal adjuvant activity via its ability to recognise certain cell-surface receptors and thus aid in the delivery of antigen to professional antigen presenting cells.

The titres of anti-peptide IgG antibodies in the sera following intranasal administration of CPMV-MUC1(16) were approximately the same when the CVP was administered with or without CT (1:53,440 with CT, 1:57,680 without CT, see Fig. 7A). In addition, high titres of anti-MUC1 IgA antibodies in intestinal lavages were found in animals immunised with or without CT (see Fig. 7B).

The conclusion is that CT is not necessary to induce mucosal or systemic immune responses to CPMV-MUC1(16) administered via a nasal route. Furthermore, CT does not enhance the immune response to CPMV-MUC1(16) following intranasal administration.

5 In further experiments, ISCOMs were used as intranasal adjuvants in place of CT. Mice were immunised with 4 doses of 100µg CPMV-MUC1(16) + 10µg ISCOM matrix. On days 42, high titres of CPMV- and MUC1-specific IgG were detected in sera, and specific IgA in bronchial, intestinal and, to a lesser extent, vaginal lavages (Figure 7C). The titres obtained with ISCOMs, however, were not significantly higher than those obtained without any exogenous adjuvant.

Example 9 – Th1 bias

10 In mice, Th1 cells mediate macrophage and cytotoxic T cell activation and B cell class-switching to the IgG_{2a} sub-class [Mosmann *et al.* (1986) *J. Immunol.* 136:2248-2357]. IgG_{2a} is the principal effector isotype of IgG that induces antibody-dependent cell cytotoxicity (ADCC) and phagocytosis, and better protects mice against tumours [Kaminski *et al.* (1986) *J. Immunol.* 136:1123-1130]. Conversely, Th2 cells induce B cells to produce IgG₁, which is
15 ineffective at mediating ADCC or phagocytosis. In view of the opposing functions of Th1 and Th2 CD4⁺ cells and of the IgG isotypes generated by these subsets, it is desirable for cancer vaccines to prime Th1 cells.

The isotype of MUC1-specific antibodies was examined in mice immunised with two doses of 100µg CPMV-MUC1(16) without adjuvant, or two doses of 10µg or 100µg CPMV-MUC1(16)
20 in QS-21. Titres of all isotypes were present, but IgG_{2a} and IgG_{2b} were predominant over IgG₁. This bias towards a Th1-type response was most pronounced in the absence of adjuvant. The control mice, immunised with KLH-MUC1 in QS-21, produced predominantly IgG₁.

The bias of the isotype response appear to be related to the ability of the virus to prime virus-specific Th1 cells which produce IFN-γ but little IL-4, allowing class-switching of
25 peptide-specific B cells to IgG_{2a}-producing plasma cells.

Example 10 – vaccination with CPMV-MUC1(16) causes a reduction in tumour burden

The ability of CPMV-MUC1(16) to elicit antibodies which can cause regression of tumours expressing the MUC1 protein was demonstrated in a mouse tumour model. Tumour cells expressing the MUC1 protein were injected subcutaneously into mice which had previously
30 been immunised with CPMV-MUC1(16). Full details are described below.

Nine C57BLScSn mice were immunised subcutaneously with 100 µg of CPMV-MUC1(16) on day 0 and 21 using Quil A (10 mg/dose) as adjuvant. A further 6 mice were immunised with phosphate buffered saline containing QuilA (PBSA) in a similar manner. On day 42 all mice were administered with 2×10^5 MUC1 RMA cells by subcutaneous injection in the flank and the tumour size measured in two dimensions over the following month using vernier calipers. Tumour volumes were calculated as $(a \times b^2)/2$ where a represents the largest diameter and b the smallest diameter. Mice were sacrificed when tumours reached ~ 1 to 1.5 cm in diameter. The results are summarised below:

Vaccine	Mouse No.	Days post challenge						
		9	14	16	21	23	28	30
CVP	1	0.108	0.06	0	0	0	0	0
	2	0.0108	0.06	0.032	0.032	0.032	0.013	0.004
	3	0.05	0.05	0	0	0	0	0
	4	0.062	0.032	0	0	0	0	0
	5	0.062	0.062	0.062	0.0864	1.14	1.8	
	6	0.108	0.126	0.196	0.6	0.85	1.617	
	7	0.126	0.126	0.441	0.441	0.793	1.68	
	8	0.126	0.126	0.108	0	0	0	
	9	0.075	0.148	0.22	1.27			
PBSA	10	0.126	0.5	0.55	1.68			
	11	0.108	0.428	0.5	1.37			
	12	0.17	0.256	0.936				
	13	0.075	0.5	0.55	1.37			
	14	0.0907	0.108	0.108	0.256	0.5	1.372	
	15	0.171	0.5	0.726	1.47			

It can be seen from the above data that over 50% of the mice (5/9 mice) immunised with CPMV-MUC1 were protected compared to 0% (6/6) in the PBSA-immunised control group.

Example 11 – further insertion points in VP-S of CPMV

Examples 1-10 illustrate the presentation of a tumour-associated mucin at the βB-βC insertion site of the CPMV S subunit. Alternative insertion sites within the S subunit are also suitable for achieving the CVPs of the present invention. In this respect, knowing the nucleic acid sequence encoding the S subunit and a range of desired insertion locations therein, the skilled person can simply prepare a suitable expression vector in a manner analogous to the preparation of pCP7.

Similarly, knowing the nucleic acid sequence encoding the L subunit of CPMV and a range of desired insertion locations therein, the skilled person can simply prepare an expression vector encoding, *inter alia*, the L subunit in a manner analogous to the preparation of pCP7.

Although examples 1 to 10 employ the MUC1(16) and MUC1(23) epitopes, any mucin peptide
5 epitope sequence can be used.

As mentioned above, a mucin peptide (*e.g.* the 16-mer) can be inserted in the $\beta C'\beta C''$ loop of the small coat protein of CPMV. For example, the peptide can be cloned in between D₄₄+D₄₅, in a suitable vector. This results in a good viral yield with good immunological properties.

Insertions in the $\beta C'\beta C''$ loop can also be combined with insertions in the C-terminus of the
10 VP-S. The C-terminus of VP-S sticks out from the surface of the virion and is highly immunogenic. The region that is not part of the "body" of the virus starts with Pro₁₈₂ and runs up to the C-terminus Ala₂₁₃. Insertions of mucin epitopes can be made in this region, with a particularly effective insertion site being between R₁₉₉ and S₂₀₀.

Example 12 – insertions in SBMV

15 The following examples illustrate the application of the present invention to plant viruses other than CPMV – any icosahedral plant virus can be used as a potential carrier for these mucin peptides. This example describes the use of SBMV instead of CPMV as a carrier for the MUC1(16) epitope.

Inspection of the crystal structure of SBMV strain C reveals that a portion of the loop between
20 the βH and βI strands is well exposed upon the surface of the virus at the five-fold and quasi-six fold axes. This portion of the loop comprises amino-acids 251 to 255 of the linear coat protein sequence and nucleotides 3967 to 3981 of the genomic RNA sequence.

The cDNA of the complete 4194bp RNA genome of SBMV is cloned into a derivative of pBluescriptII plasmid vector lacking the T7 and T3 promoters using standard techniques. The
25 cDNA is cloned immediately downstream of a bacteriophage T7 such that a unique restriction enzyme site is present at the 3' terminus of the cDNA, thus allowing linearisation of the recombinant plasmid to generate run-off transcripts which mimic the wild-type RNA. As an alternative, the cauliflower mosaic virus (CaMV) 35S promoter may be used.

A sub-clone is then made from this full-length cDNA clone by inserting the *BglII-XmnI*
30 fragment (genomic RNA nucleotides 3165-4161), which contains within it the whole coat

protein open reading frame, into *Bam*HI/*Hinc*II digested pBluescriptII. This sub-clone is further manipulated via site-directed mutagenesis at genomic nucleotide positions 3969 (change A to C) and 3984 (G to T) to create *Bam*HI and *Hpa*I restriction sites, respectively (Figure 8A). The modified subclone is digested with these enzymes and separated from the small excised
5 fragment which is replaced by oligonucleotides coding for the excised nucleotide sequence plus nucleotides coding for the MUC1(16) insert. The five constructs shown in Figure 8B contain the insert between coat protein amino acids 251-252, 252-253, 253-254, 254-255, or 255-256.

The modified region of the coat protein from each of these constructs is isolated on a *Hind*III/*Avr*II fragment (genomic nucleotides 3434-4096) and used to replace the corresponding
10 fragment in the full-length cDNA clone of the virus. Each of these clones is then linearised at the 3' terminus of the cDNA and, in the case of a T7 bacteriophage promoter construct, used to generate capped run-off RNA transcripts which are then inoculated onto the host-plant (*Vigna unguiculata*), or inoculated directly when under the control of the 35S promoter.

The inoculated plants are monitored for symptoms, and the strength of symptoms, yield and
15 stability for each construct are assessed in order to determine the optimal insertion site. If desirable, purified virus may also be used to immunise experimental animals in order to determine the levels of immune response generated by each construct.

This example can be extrapolated to allow insertion in any of the exposed loops of SBMV. Similarly, any mucin peptide epitope sequence can be used instead of MUC1(16).

20 ***Example 13 – insertions in LTSV***

This example describes the determination of a potential insertion site for epitopes by alignment of the primary sequence of a virus whose structure is unknown (LTSV), against those of viruses whose structure has been determined.

The crystal structures of two sobemoviruses (SBMV and SMV) have been solved at high
25 resolution. Comparison of the crystal structures reveals that secondary structural elements are well conserved between the viruses and, in particular, the protruding loop between the β H and β I is almost identical in shape and location between the two viruses. This structural element would therefore be expected to be well conserved in all sobemoviruses.

Alignment of the primary sequences of LTSV, SBMV and SMV shows a strong conservation of
30 residues between the three viruses within the β H strand region and significant sequence

homology within the β I strand (Fig.9). This allows the loop region of LTSV to be inferred as spanning amino acids 218 to 224 of the coat protein.

The 4275bp LTSV RNA genome is cloned as cDNA, as described for SBMV in example 12. The genomic clone is then modified by site directed mutagenesis at position 3959 (C to T) and position 3998 (T to C) to create unique *Pst*I and *Kpn*I restriction enzyme sites, respectively (Fig.10A). The modified genomic clone is digested with these restriction enzymes and separated from the small excised fragment which is replaced by oligonucleotides coding for the excised nucleotide sequence plus nucleotides coding for the epitope sequence MUC1(16). The six constructs shown in Fig.10B contain the epitope sequence between coat protein amino acids 218-219, 219-220, 220-221, 221-222, 222-223, or 223-224.

Each of these clones is linearised at the 3' terminus of the cDNA and, in the case of a T7 bacteriophage promoter construct, used to generate capped run-off RNA transcripts which are then inoculated onto the host-plant (*Nicotiana clevelandii*), or inoculated directly when under the control of the 35S promoter. The inoculated plants are used as described in example 12.

This example can be adapted to insert of any peptide into any of the exposed loops of LTSV.

Example 14 – insertions in RCNMV

Like example 13, this example describes the determination of potential insertion sites in a virus (red clover necrotic mosaic virus, RCNMV), whose crystal structure is unknown, using primary structural alignments with a second virus whose crystal structure has been determined (TBSV).

This example uses secondary structure prediction algorithms.

The crystal structure of the coat protein of TBSV reveals that each of the 180 coat protein subunits forming the T=3 icosahedron consists of two β -barrel domains. The first domain forms the surface of the virus particle and is termed the S domain and is equivalent to the single domain found in SBMV. The second, much smaller, domain forms a surface protrusion at right angles to the plane of the S domain. This P domain forms dimeric interactions with the P domain of a neighbouring coat-protein subunit at the strict and quasi two-fold axes of the icosahedron. The presence of the P domain causes the virions to appear distinctly granular when examined under the electron microscope. Between the S and P domains is a short flexible linker followed by a pair of β -strands connected by a loop which appears to be highly exposed on the viral surface with no obvious role in the contacts between subunits. This loop provides a potential target for epitope insertions.

Dianthoviruses (*e.g.* RCNMV) also appear distinctly granular when subjected to electron microscopy, and this together with the size of the coat proteins and their limited homology with those of tombusviruses suggests that they may have structural similarity. Alignment of the coat protein sequences of RCNMV and TBSV (Fig.11) using a Lipman-Pearson alignment algorithm, which recognises sequence conservation as well as identity, gives a similarity index of 26.9 (strict homology is 23%). From the alignment it can be seen that the S domain is better conserved (TBSV residues 100 to 269, strict homology 36%) than the P domain (TBSV residues 270-388, poorly conserved).

The loop of interest comprises TBSV residues L²⁸⁰A²⁸¹G²⁸² and the sequence around this region shows some similarity to the sequence of RCNMV. Secondary structure prediction algorithms are also used to predict the location of β -strands, however, and hence the loops which lie between them. Fig.12 shows a Chou-Fasman β -region prediction plot of RCNMV residues 214-254 using an algorithm based upon the structures found in 64 proteins which is claimed to be 80% accurate at predicting β -strands of interest. The plot suggests that β -strands of interest are located between residues 214-221 and 226-228, and hence the loop at the tip of the domain will be residues 222-225 and residues 245-248. A more sophisticated prediction algorithm, the EMBL PHDsec program based upon trained neural networks, may also be used. The resulting output for the region of interest is shown in Fig.13. This locates the β -strands to residues 220-223 and 227-239, therefore the loop is comprised of residues 224-226. Combining the two sets of data, the loop will lie within the region spanned by residues 222 to 226.

Dianthoviruses have a bipartite RNA genome, both RNAs being required for infectivity. Accordingly, RCNMV RNAs 1 and 2 are cloned as cDNA, using standard molecular biological techniques, into a suitable vector, downstream of a CaMV 35S promoter. As an alternative, the T7 promoter may be used. Both clones are engineered such that they can be linearised at the 3' termini of the cDNAs.

The cDNA genomic clone of RNA1 is modified by site directed mutagenesis at positions 3078 (A to G) and 3081 (G to C), to create a unique *Apa*LI restriction site, and at positions 3108 to 3111 (ACTC to GTTA) to create a unique *Hpa*I restriction site (Fig.14A). Although the mutation at position 3081 is not silent, the correct codon can be restored when ligating in oligonucleotides to generate the epitope insertion. The modified genomic clone is digested with these restriction enzymes and separated from the small excised fragment which is replaced by oligonucleotides coding for the excised nucleotide sequence plus nucleotides coding for the

MUC1(16) epitope. The six constructs shown in Fig.14B insert the epitope sequence between coat protein amino acids 221-222, 222-223, 223-224, 224-225, 225-226, or 226-227.

As in examples 12 and 13, each of these clones is linearised at the 3' terminus of the cDNA and, in the case of a T7 promoter construct, used to generate capped run-off RNA transcripts. These
5 are then mixed with similar transcripts from the linearised cDNA clone of genomic RNA2 and inoculated onto the host-plant. Linearised clones are inoculated directly when under the control of the CaMV 35S promoter. Inoculated plants are used as described previously.

This example can be adapted to allow insertions of any peptide into any exposed RCNMV loop.

It will be understood that the invention has been described by way of example only and
10 modifications may be made whilst remaining within the scope and spirit of the invention.

CLAIMS

1. A chimaeric virus particle derived from a plant virus having a coat protein with a beta barrel structure and modified by insertion of an immunogenically active peptide of a tumour-associated mucin at an immunogenically effective site in the coat protein.
- 5 2. A chimaeric virus particle according to Claim 1, in which the insert is present in a loop connecting beta sheets.
3. A chimaeric virus particle according to Claim 1, in which the insert is present in the region of the C-terminus of a coat protein.
- 10 4. A chimaeric virus particle according to Claim 3, in which the insert is present at a point within 30 amino acids, preferably within 15 amino acids, of the C-terminus.
5. A chimaeric virus particle according to any of Claims 1 to 4, in which the tumour-associated mucin is PEM.
- 15 6. A chimaeric virus particle according to Claim 5, in which the insert is a peptide derived from the 20 amino acid repeat of the extracellular portion of the MUC1 transmembrane molecule.
7. A chimaeric virus particle according to Claim 6, in which the peptide is a 16-mer, preferably SEQ ID 6, or a 23-mer peptide, preferably SEQ ID 7.
8. A chimaeric virus particle according to any of the preceding claims, in which the plant virus is a comovirus.
- 20 9. A chimaeric virus particle according to Claim 8, in which the plant virus is cowpea mosaic virus.
10. A chimaeric virus particle according to Claim 9, in which the insert is present in the S protein of the virus.
- 25 11. A method for producing a chimaeric virus particle according to any of Claims 1 to 10, which comprises introducing a nucleotide sequence coding for the tumour-associated mucin peptide to modify the plant viral nucleic acid which codes for the coat protein; infecting plants, plant tissue, plant cells, or protoplasts with the modified viral nucleic acid; and harvesting chimaeric virus particles.

12. A method according to Claim 11, in which the introduced nucleotide sequence is inserted in that part of the plant viral nucleic acid which codes for an exposed region of the coat protein.
- 5 13. A method according to Claim 11, applied to an RNA plant virus, which comprises introducing a DNA coding for the tumour-associated mucin peptide into a cDNA corresponding to the RNA of the plant virus which codes for an exposed portion of its coat protein; inoculating plants, plant tissue, plant cells, or protoplasts with the thus modified cDNA or an RNA transcript thereof, if necessary together with any other DNA or RNA required for multiplication and assembly of whole virus particles in the plant material; and
- 10 harvesting chimaeric virus particles.
14. A method according to Claim 13, in which the modified cDNA is produced by introducing the DNA encoding the mucin peptide into a DNA fragment excised from the plant viral cDNA, and reinserting the modified excised fragment so as to constitute the plant viral cDNA in modified form.
- 15 15. A method according to any of Claims 11 to 14, in which modified virus produced, or RNA extracted therefrom, is passaged in plants to produce further yields of modified virus.
16. A vaccine comprising chimaeric virus particles according to any of Claims 1 to 10 as an immunogenic component thereof.
17. A vaccine according to Claim 16, further comprising an adjuvant.
- 20 18. A vaccine according to Claim 17, in which the adjuvant is selected from Freund's complete adjuvant, QuilA, QS-21, ISCOM matrix, alum, alammulin; or combinations thereof.
19. A vaccine according to Claim 16, said vaccine being substantially free from adjuvant.
20. A method of eliciting in an animal, including a mammal, an immune response characterised by the production of serum immunoglobulins specific for mucin peptides or polypeptides,
- 25 which comprises the administration of an immunogenic complex.
21. A chimaeric virus particle according to any of Claims 1 to 10 for use as a vaccine.
22. The use of a chimaeric virus particle according to any of Claims 1 to 10 in the manufacture of a vaccine for the treatment and/or prevention of tumours and/or cancer.

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FIG. 1

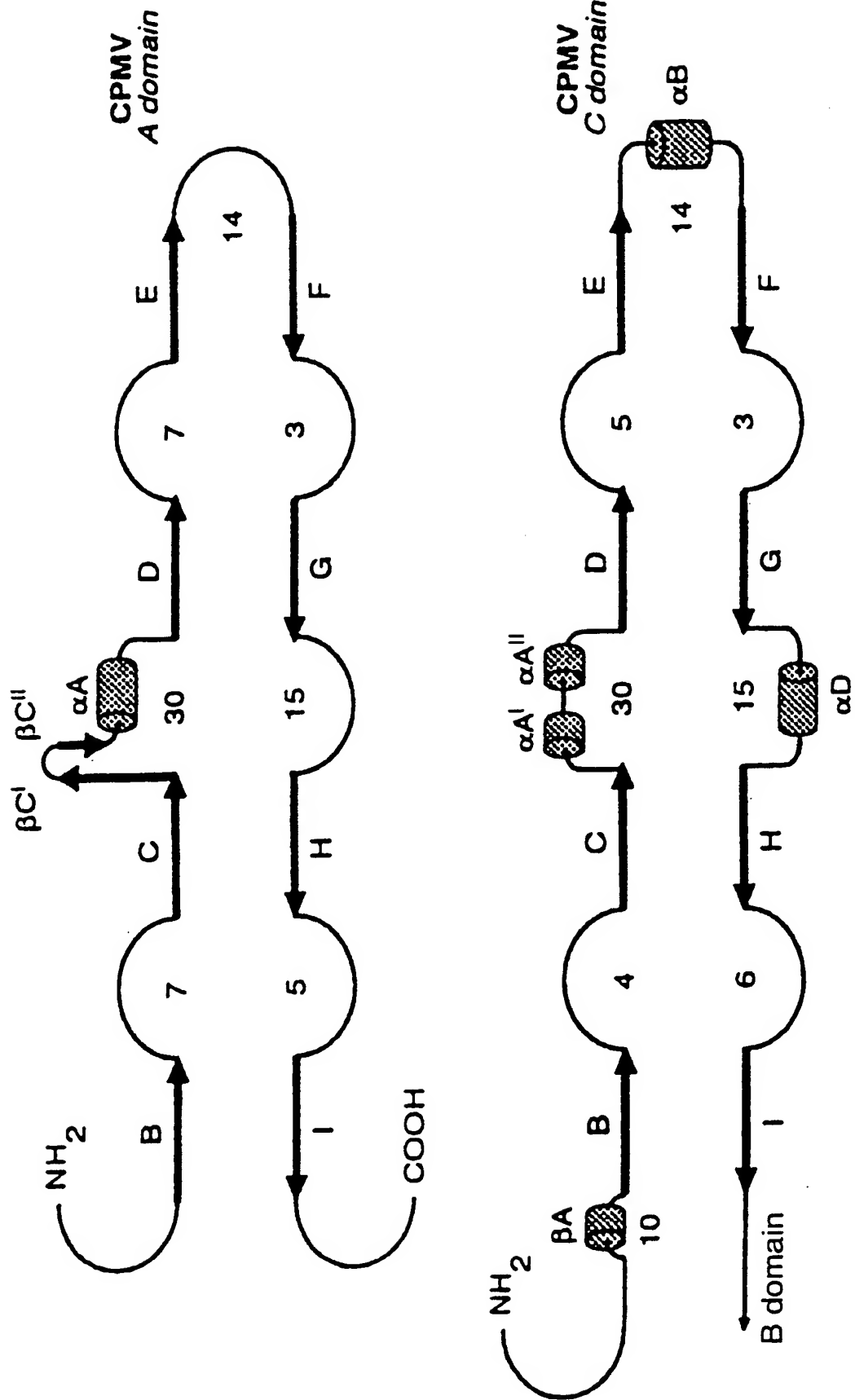
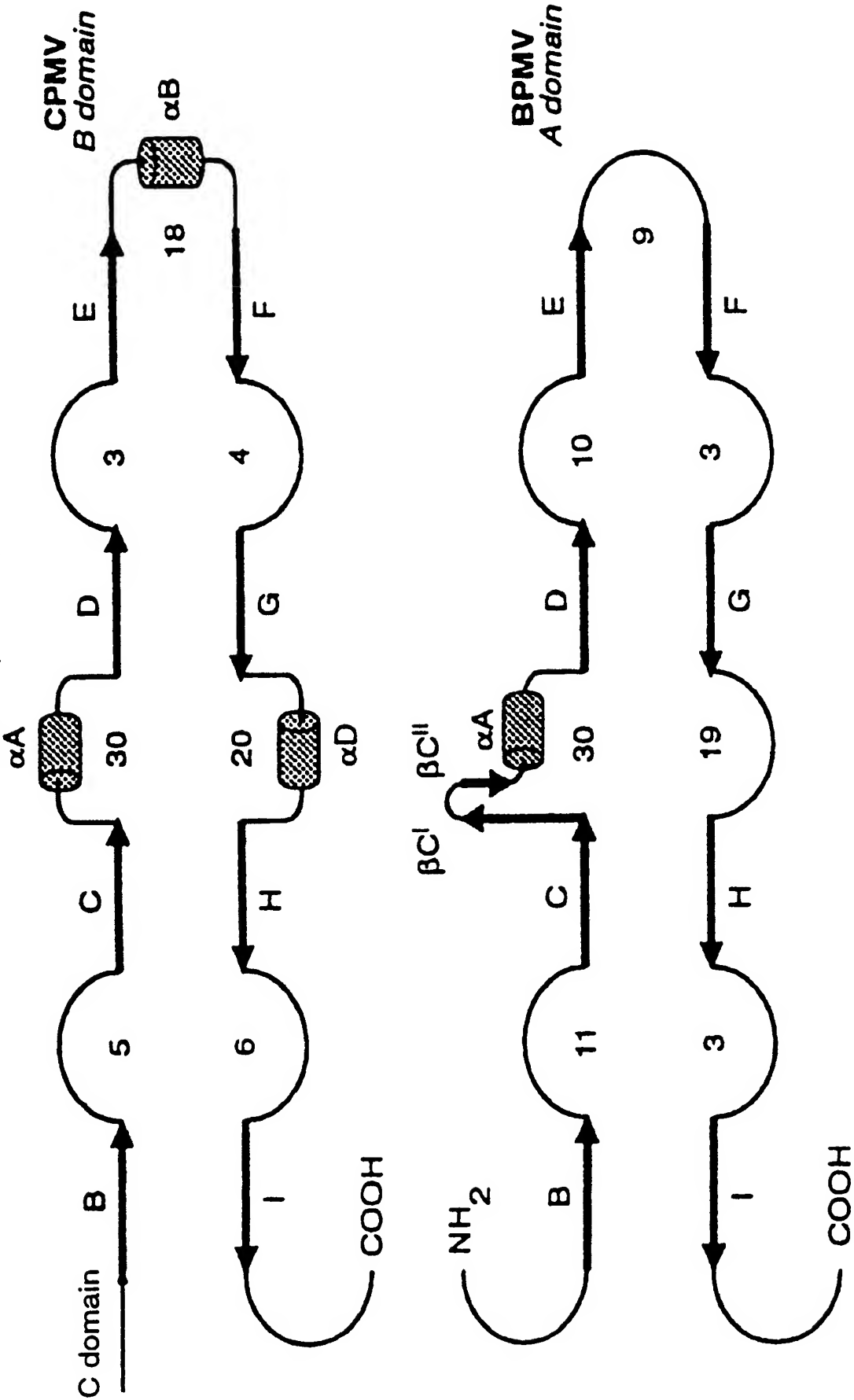
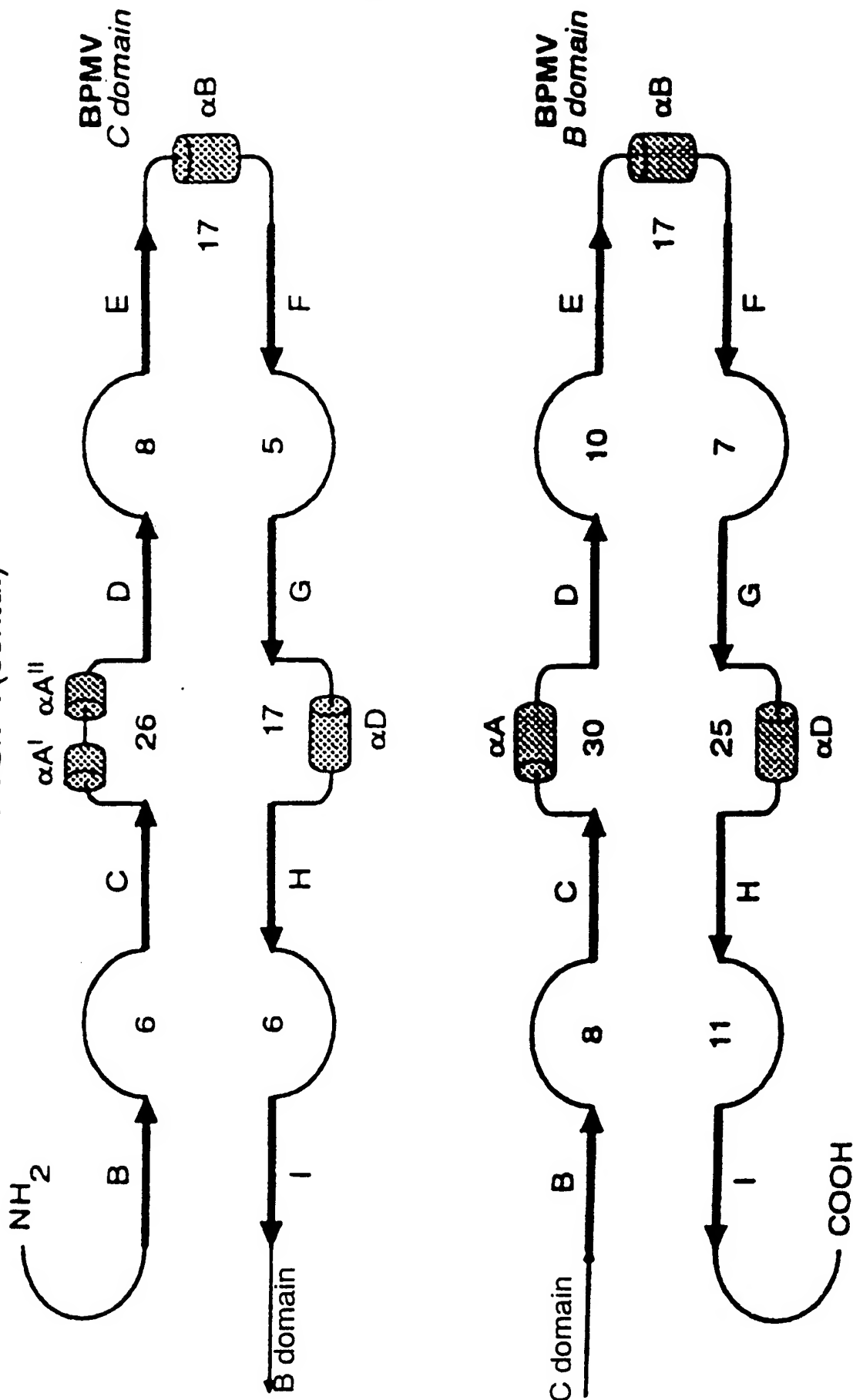


FIG. 1 (contd.)



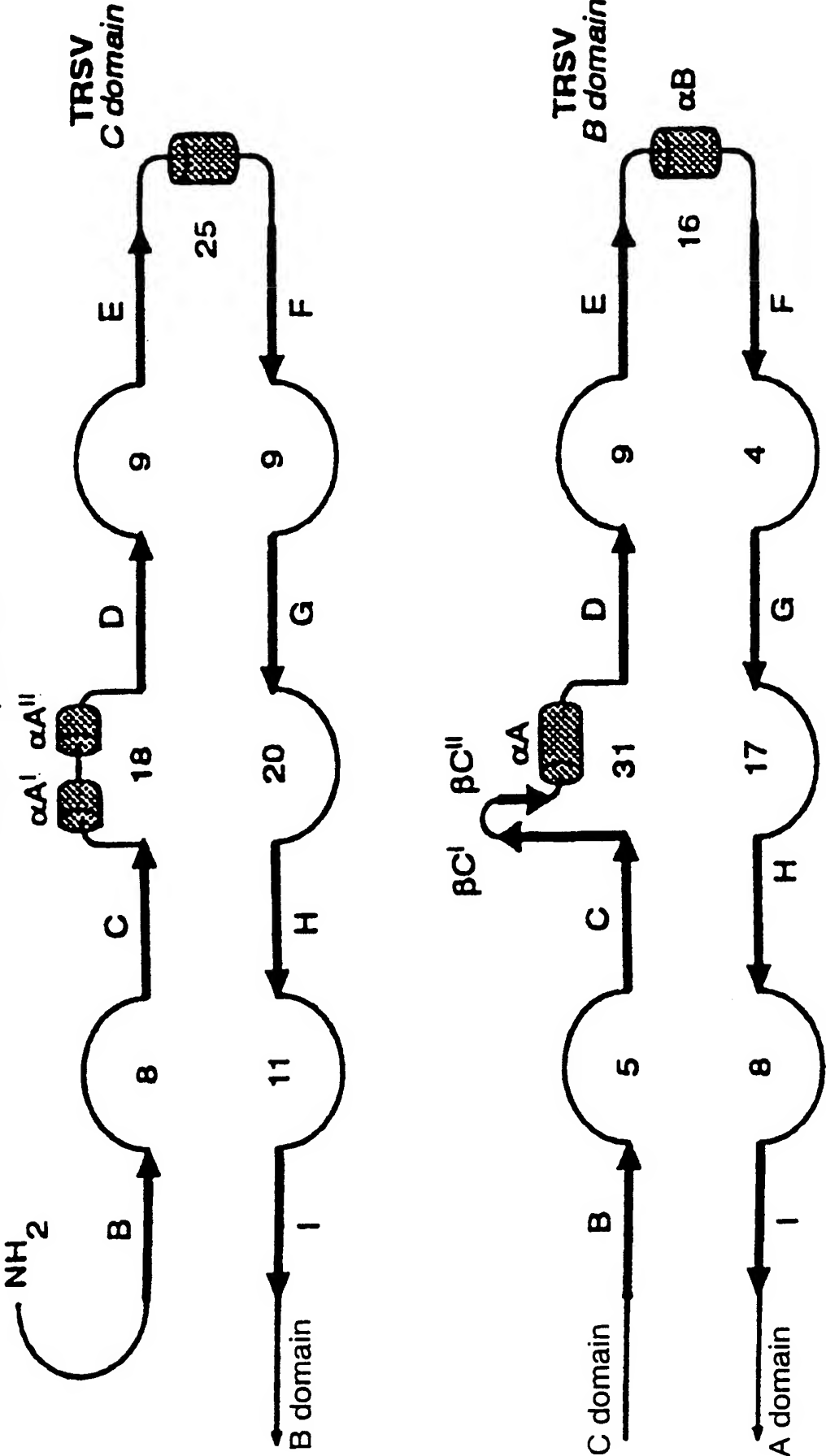
3/26

FIG. 1(contd.)



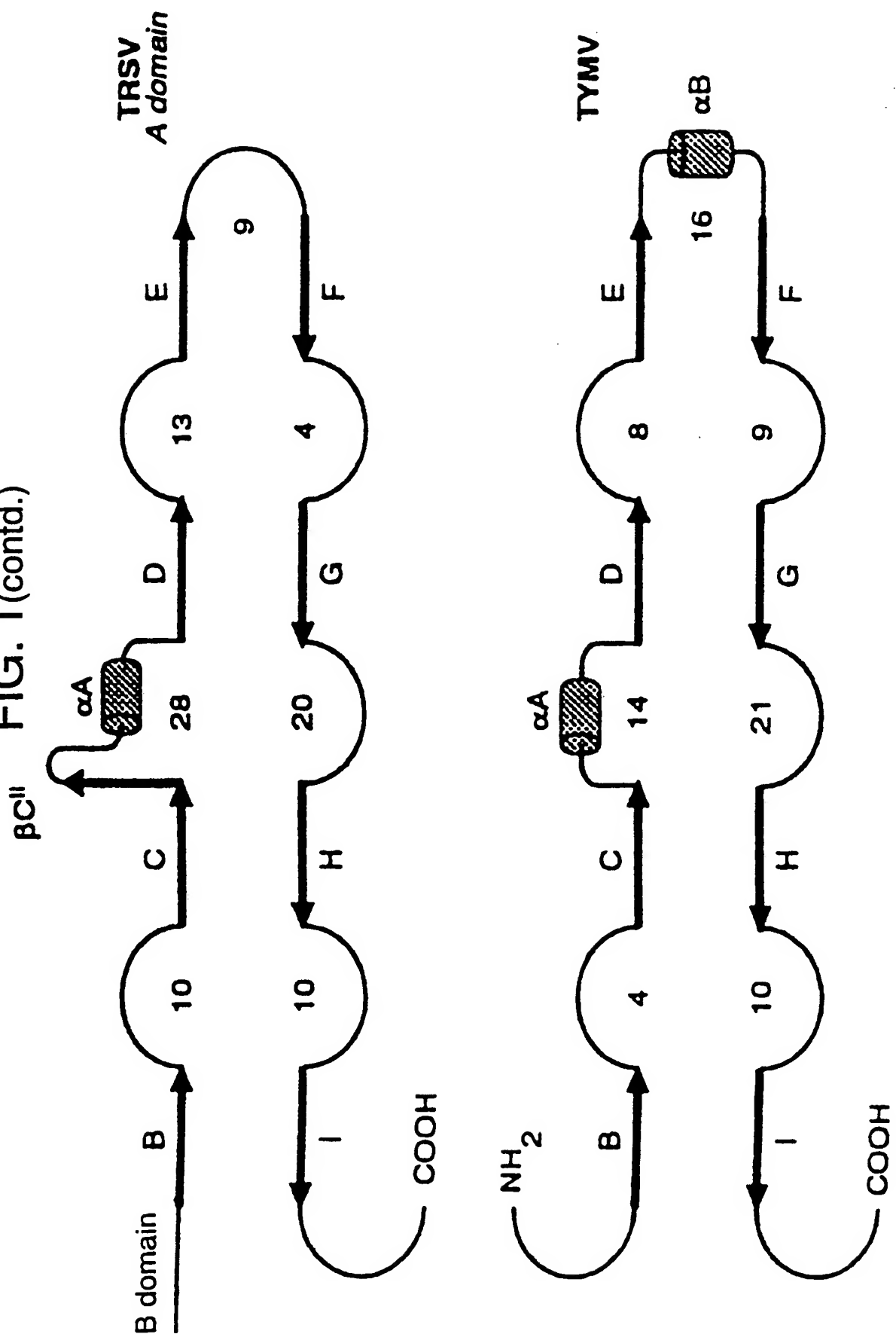
4/26

FIG. 1(contd.)



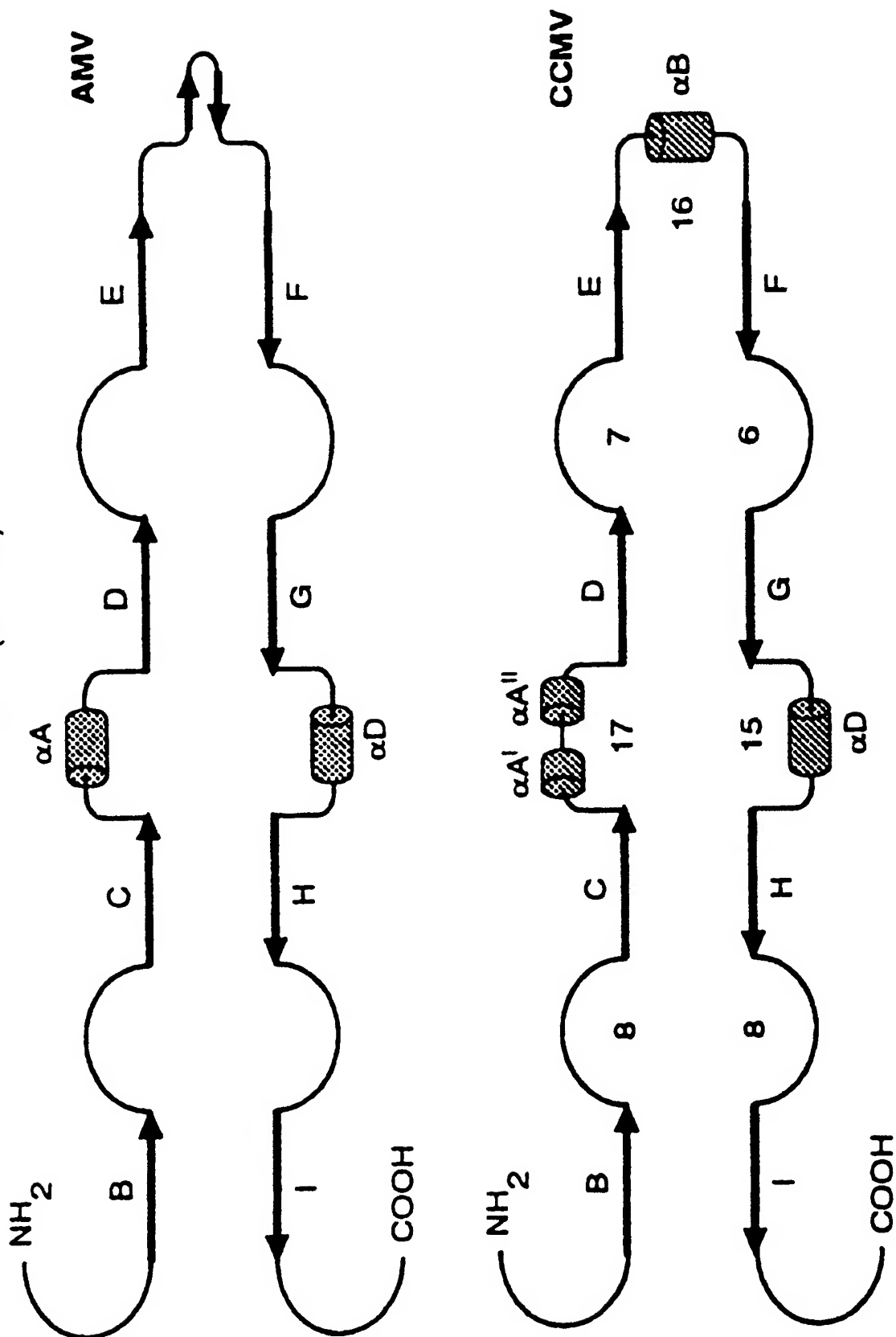
5/26

FIG. 1(contd.)



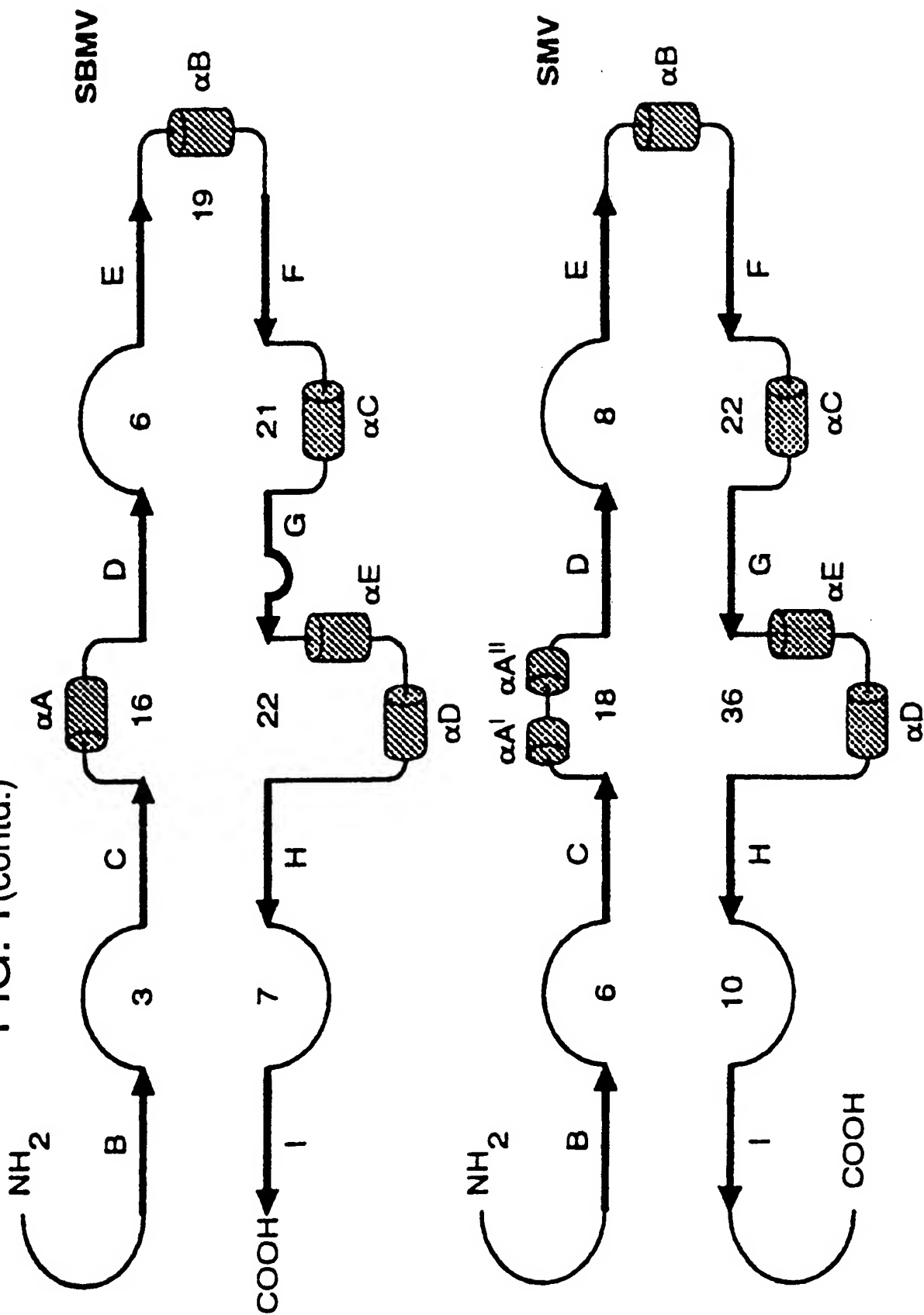
6/26

FIG. 1(contd.)



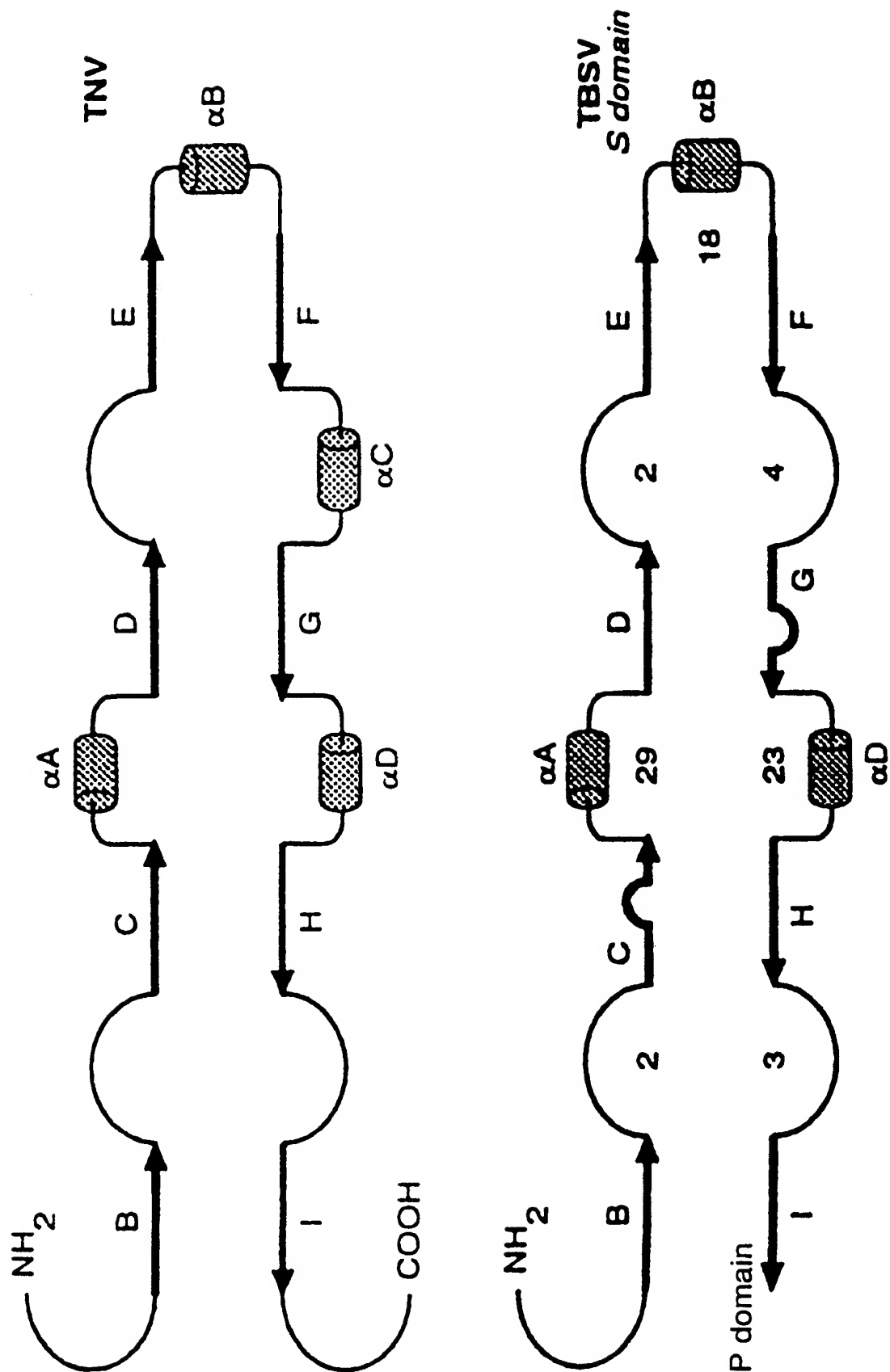
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FIG. 1 (contd.)



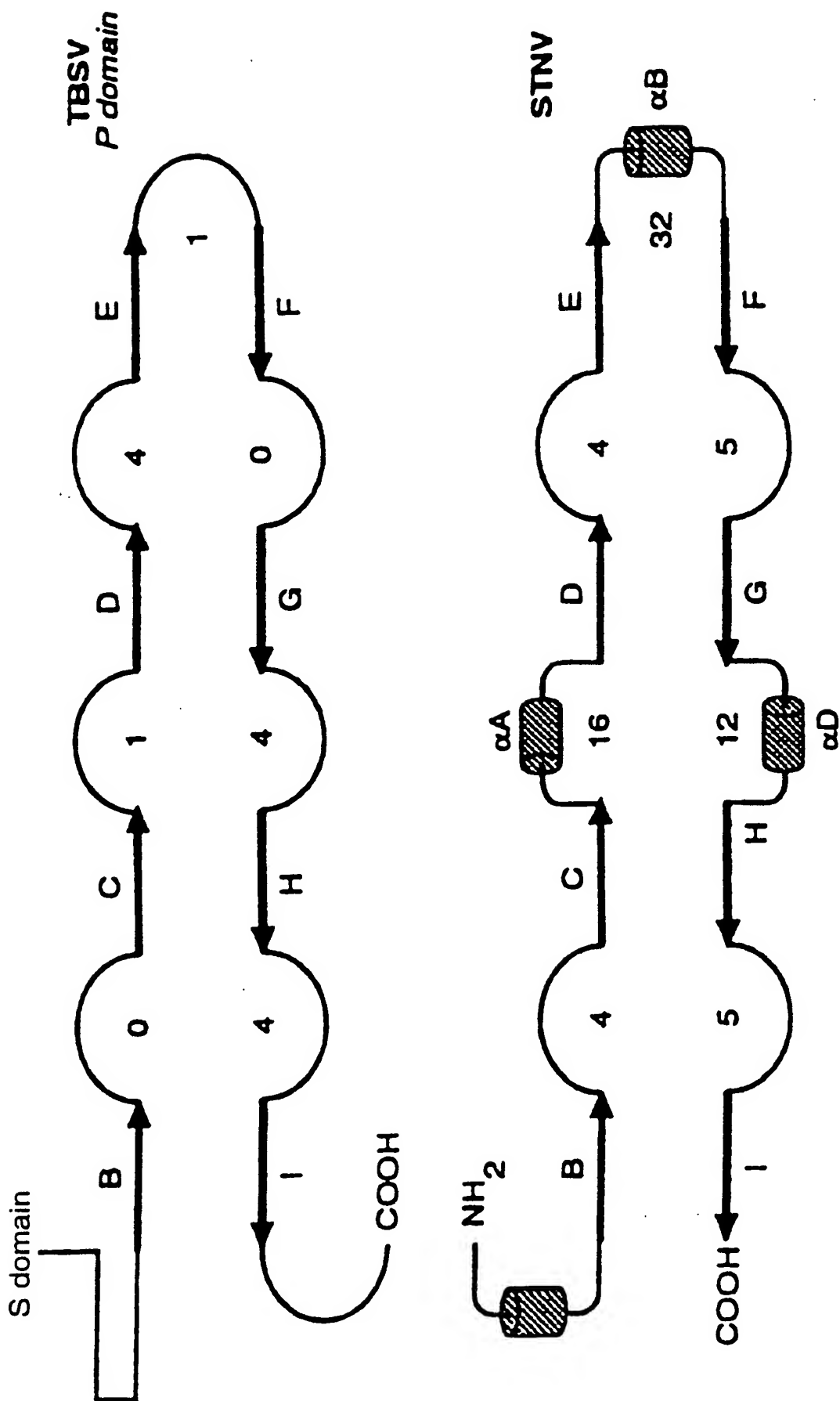
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FIG. 1(contd.)



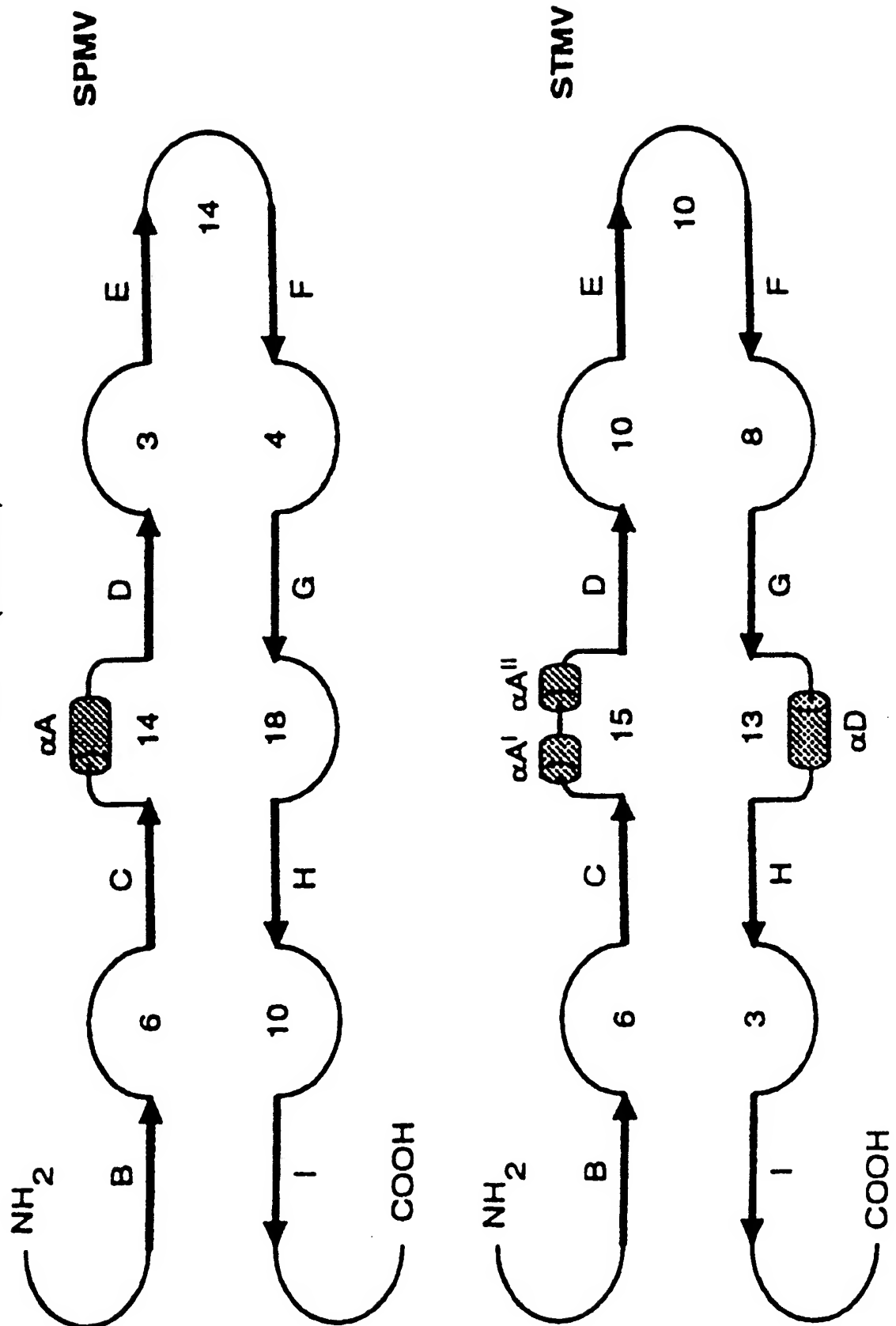
9/26

FIG. 1(contd.)



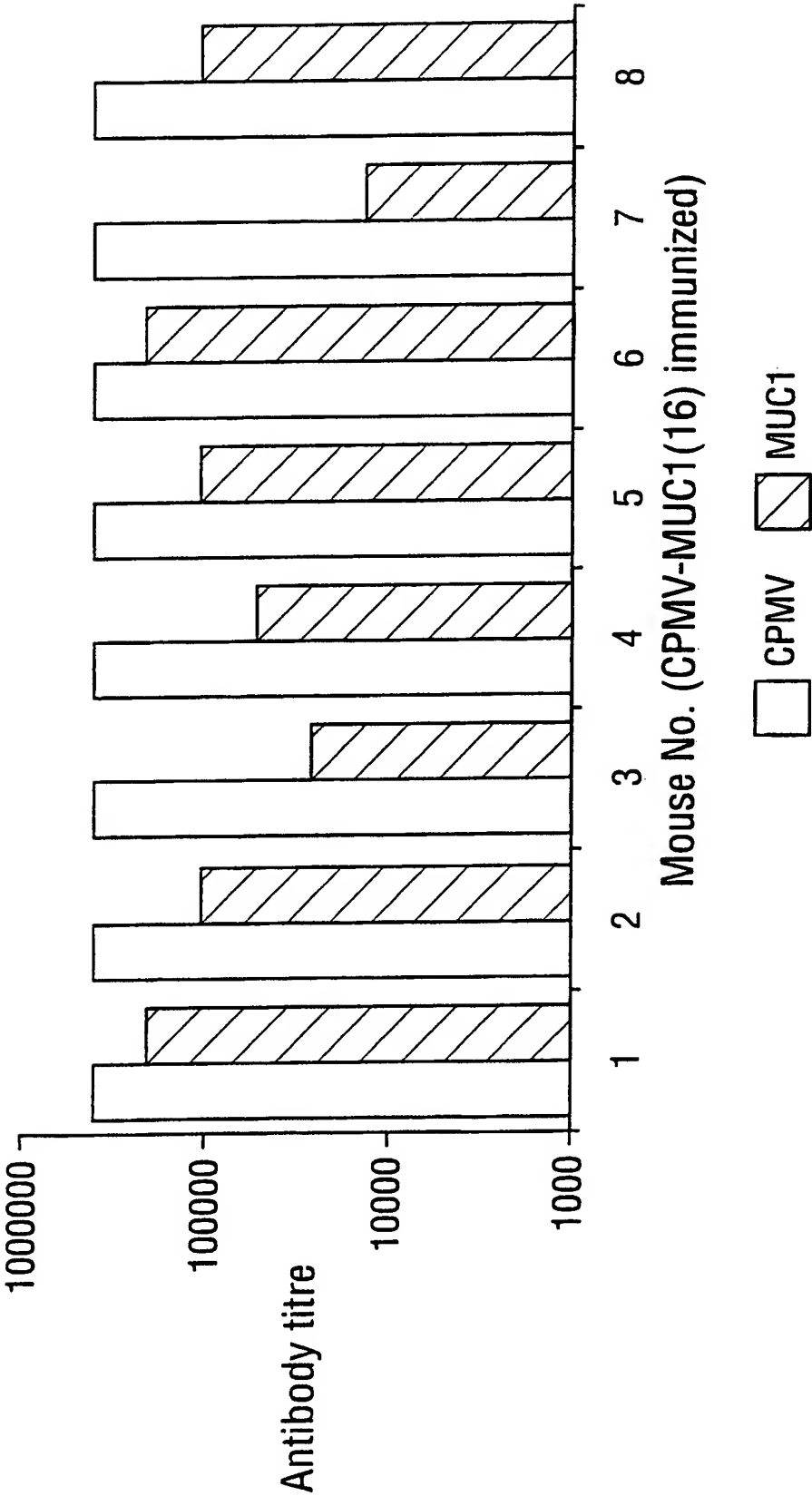
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FIG. 1(contd.)



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FIG. 2A



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FIG. 2B

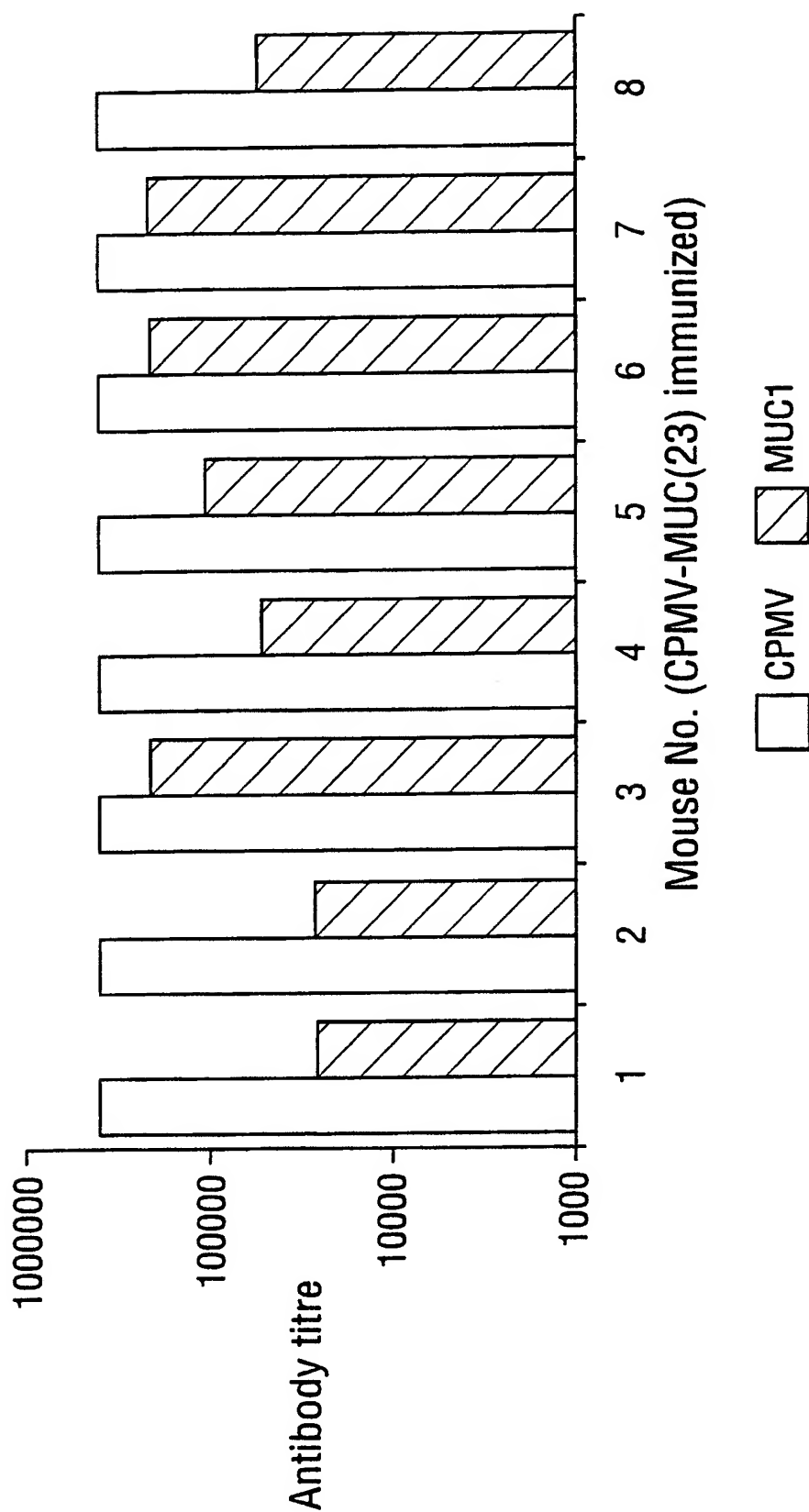
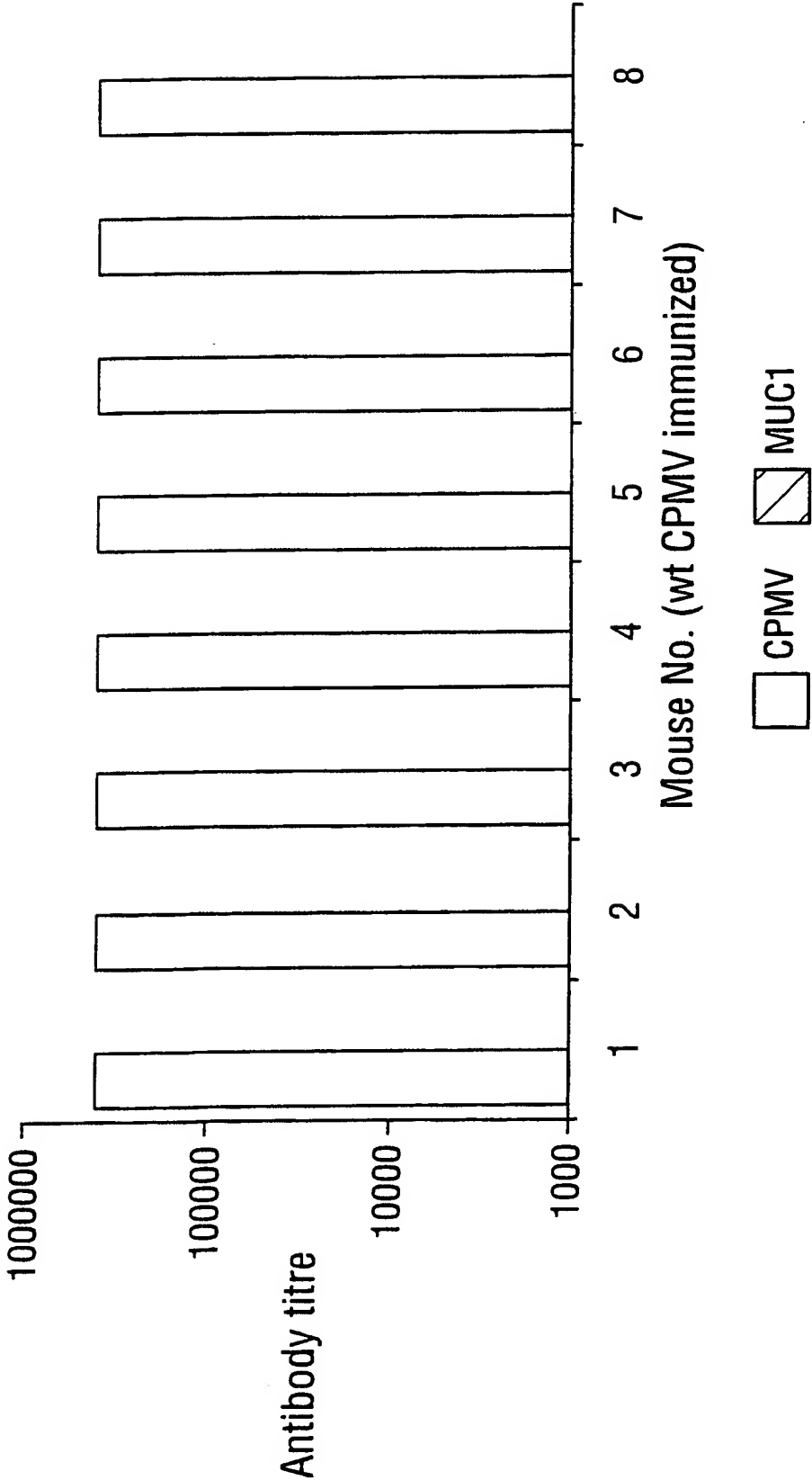


FIG. 2C



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FIG. 3A

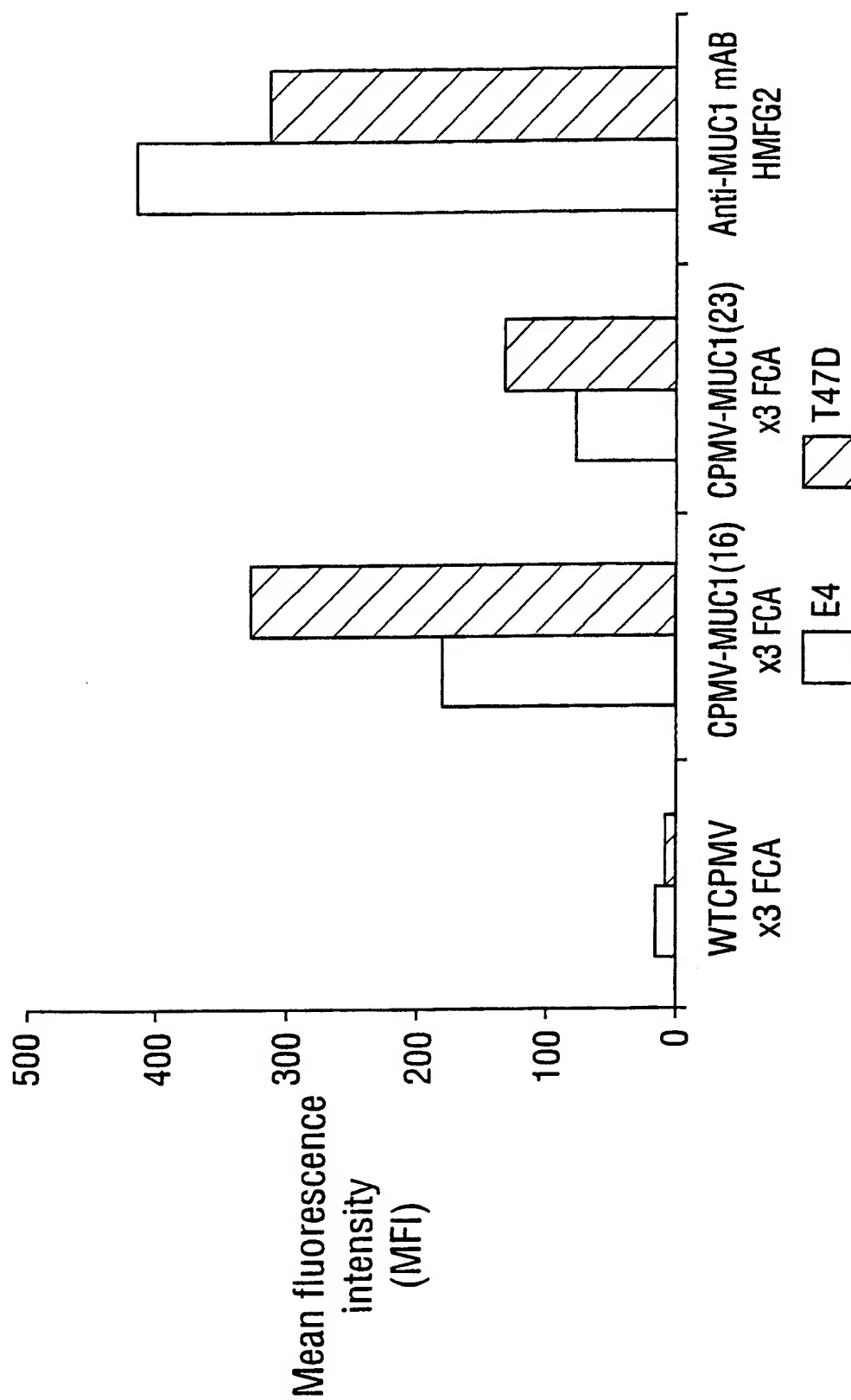
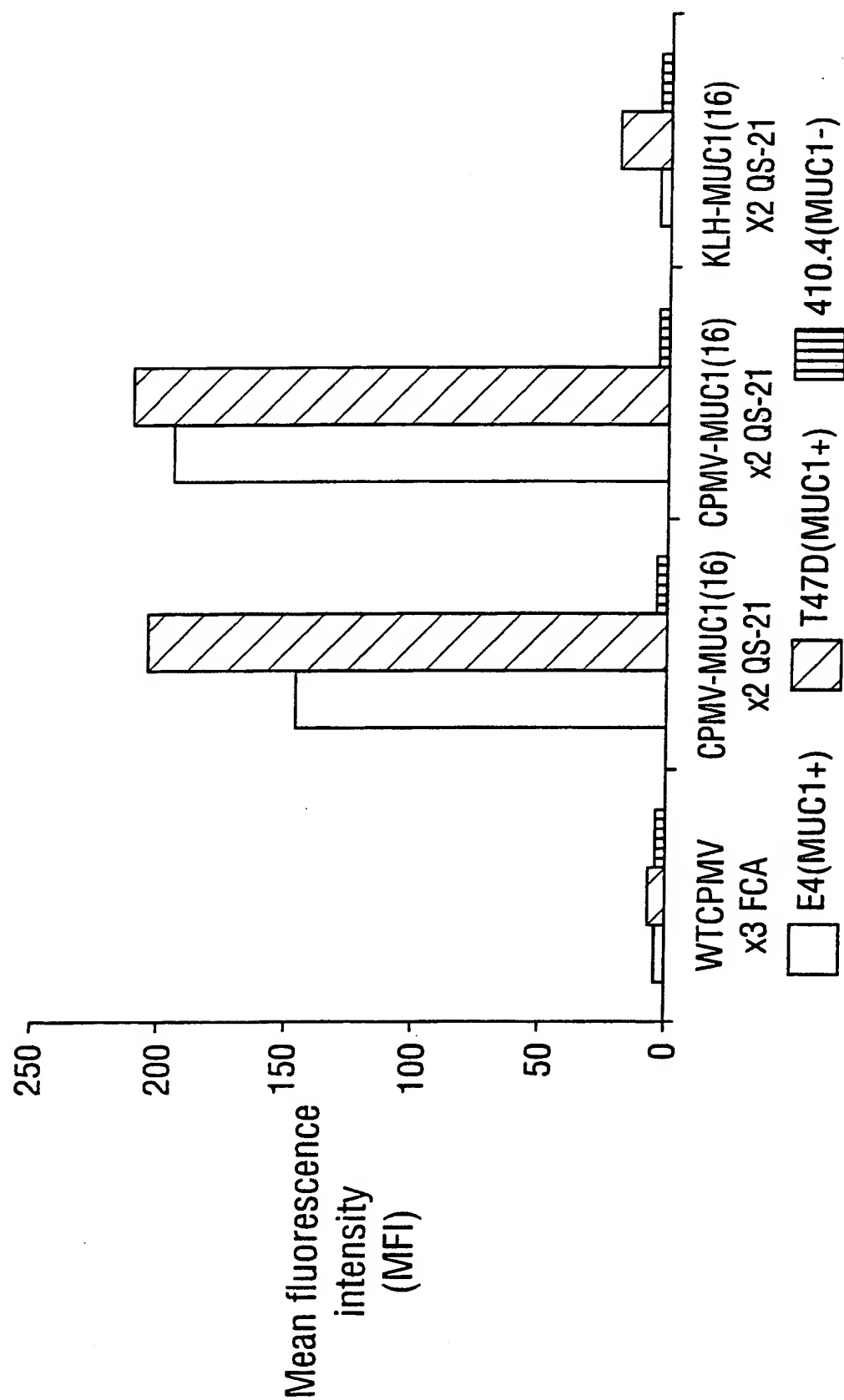
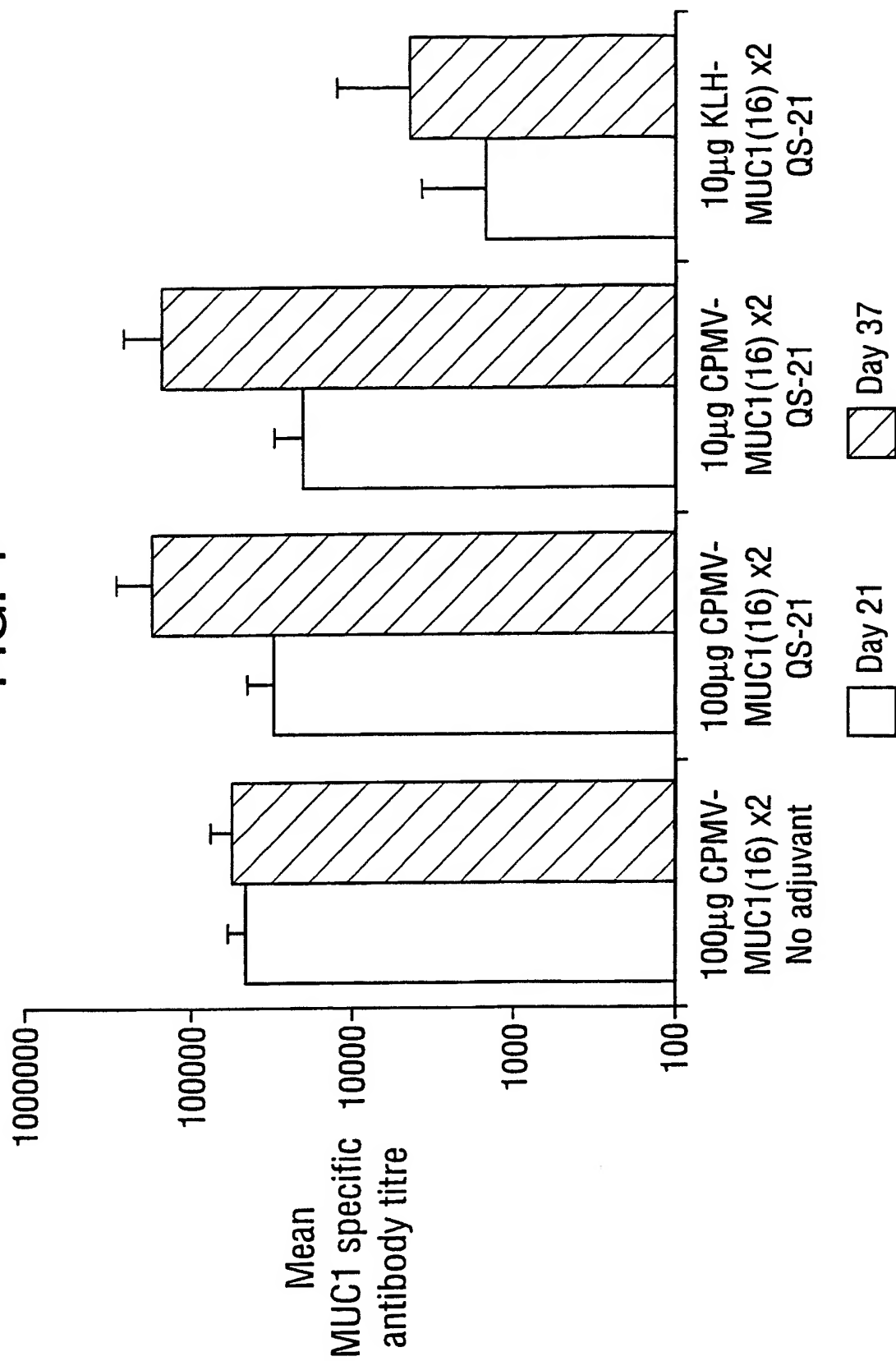


FIG. 3B



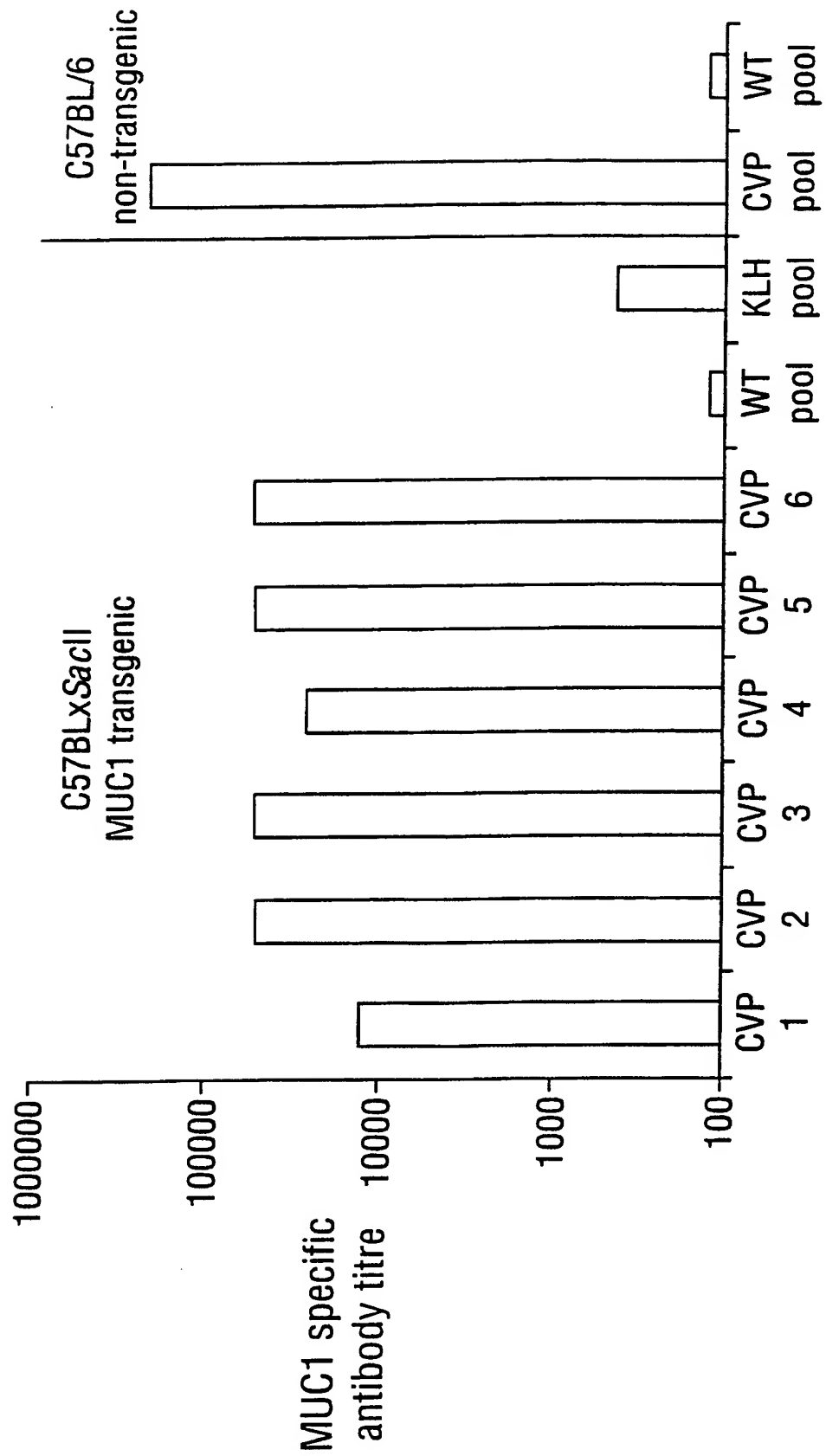
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FIG. 4



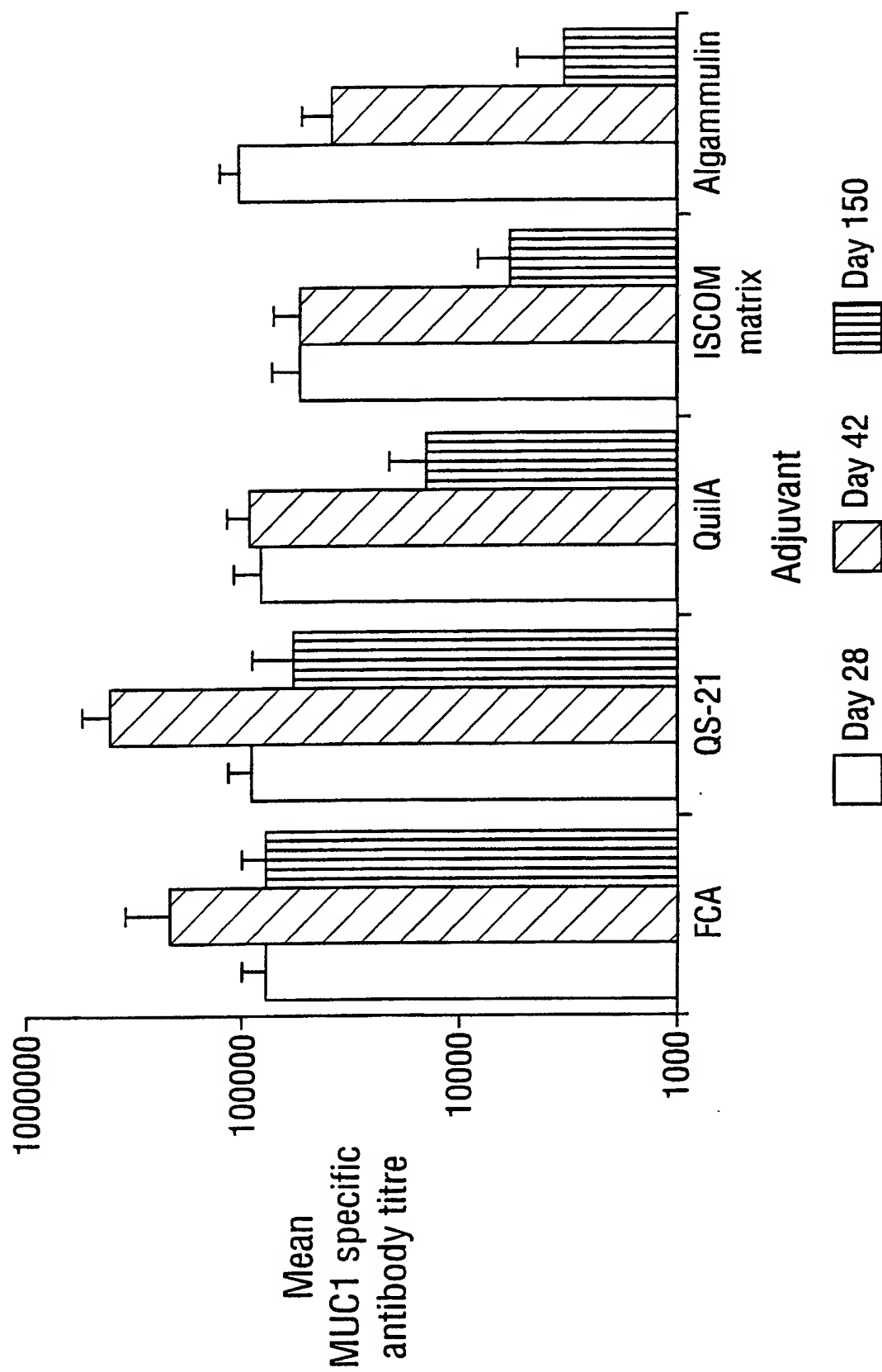
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FIG. 5



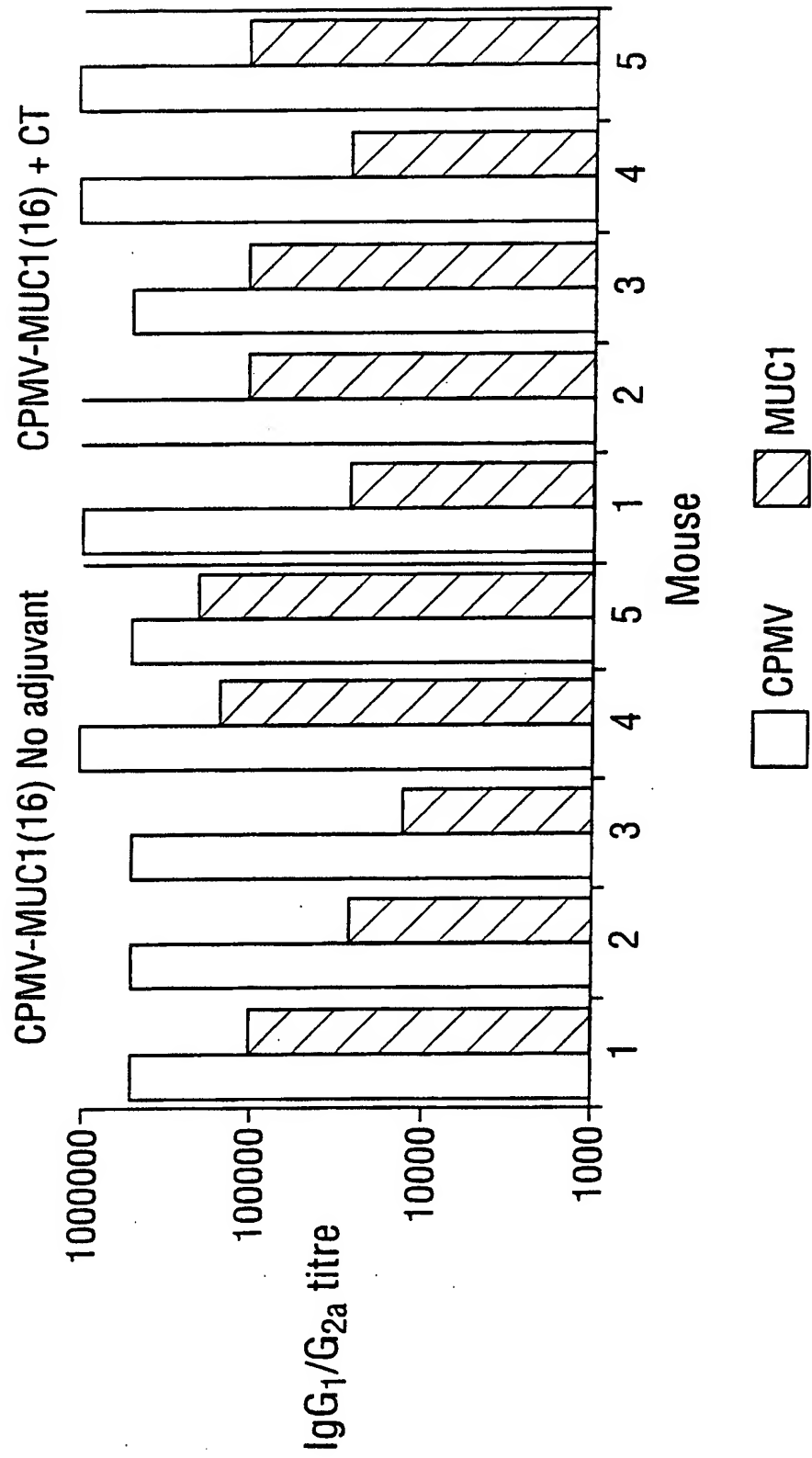
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FIG. 6



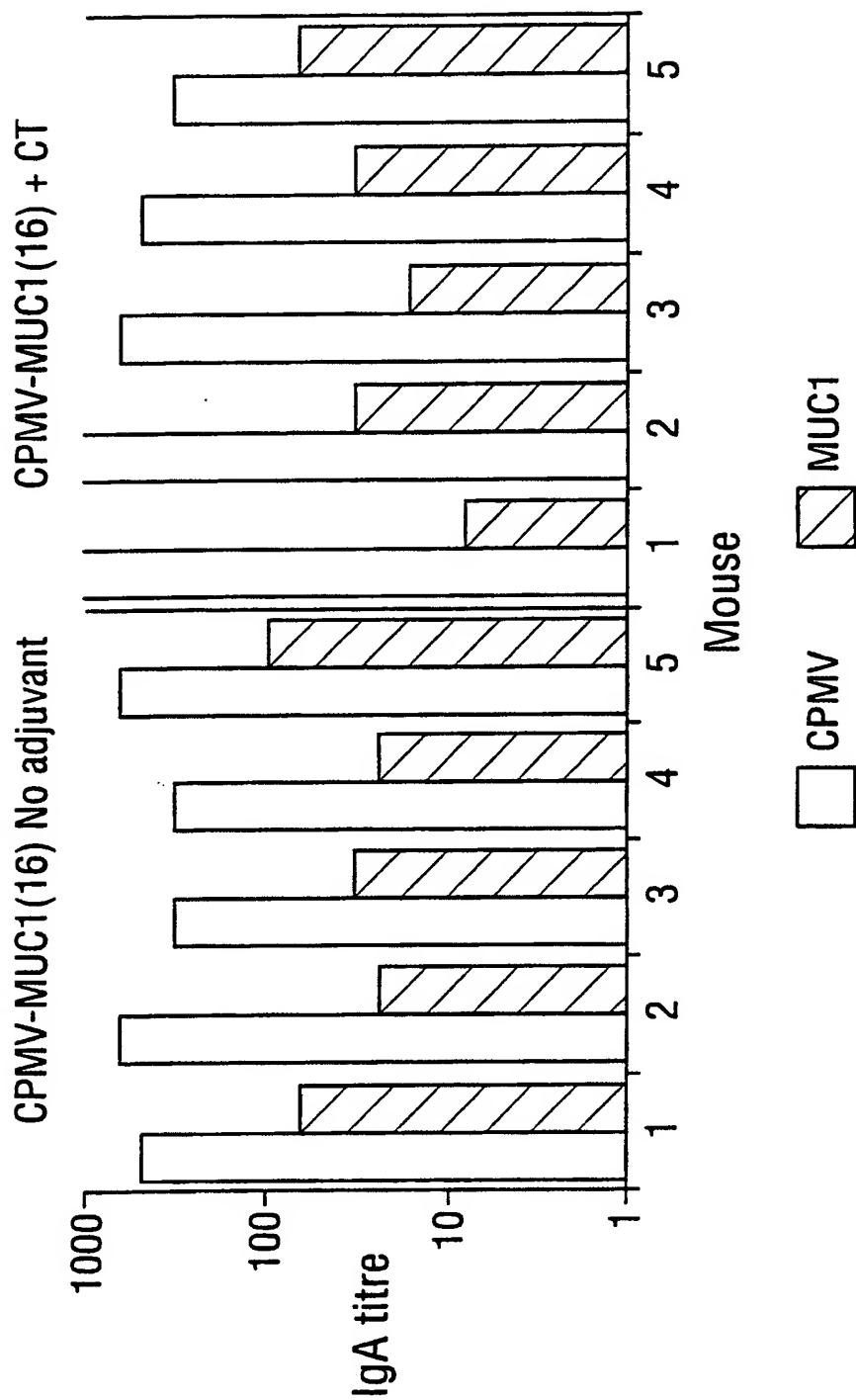
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FIG. 7A



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FIG. 7B



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FIG. 8(a)

Sequence of SBMV Coat Protein Spanning The Potential Insertion Site With Introduced Base Changes and New Restriction Sites: (sequence starts at nt 3955)

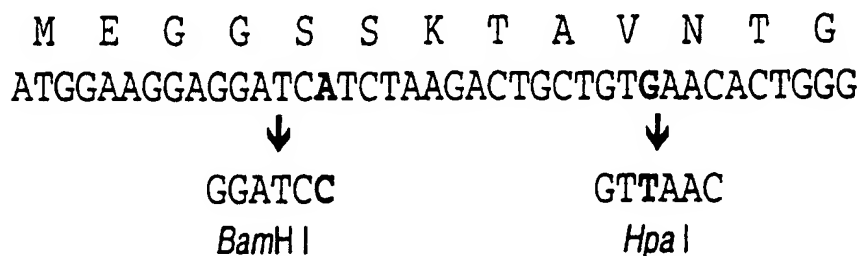
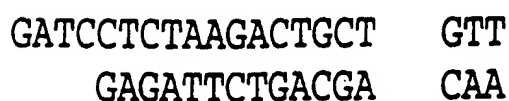
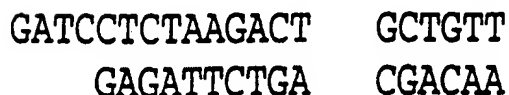
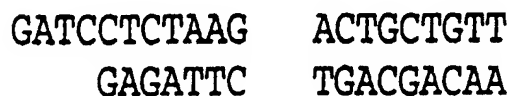
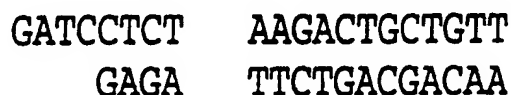
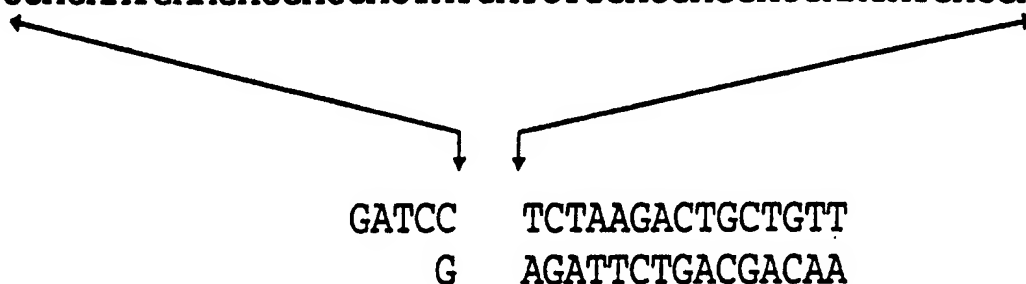


FIG. 8(b)

Series of Sequences to be Inserted Between the Restriction Sites to Insert the MUC1(16) Epitope at Various Locations

G V T S A P D T R P A P G S T A
 GGTGTTACTTCTGCTCCTGATACTAGACCTGCTCCTGGTTCTACTGCT
 CCACAATGAAGACGACCACTATGATCTGGACGAGGACCAAGATGACGA



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FIG. 9

LTSV : NI---YAPARLTIAA-ANSSINIASVGTLYATYEVEL
 SBMV : NIGNILVPARLVIAIEGGSSKTAVNTGRLYASYTIRL
 SMV : NIATDLVPARLVIALLDGSSSTAVAAGRIYASYTIQM
 #####=====#####
 β H loop β I

FIG. 13

	220	230	240
AA	ASIVQKYVIDLGGTLTSFEGPSYLMPP		
PHD sec	HHHHHEEEEE EEEE EEEEE		
Rel sec	145432244525515625586487624		
detail :			
prH sec	46665532111110000000000000		
prE sec	101123456632246752212688753		
prL sec	422221112246642237787311246		
subset : SUB sec	..H.....E.LL.EE.LLLL.EEE..		

Abbreviations :

AA : amino acid sequence

H : helix

E : extended (sheet)

blank : other (loop)

PHD : Profile network prediction HeiDelberg

Rel : Reliability index of prediction (0-9)

prH : probability for assigning helix

prE : probability for assigning strand

prL : probability for assigning loop

SUB : a subset of the prediction, for all residues with an average expected accuracy of >82%

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FIG. 10(a)

Sequence of LTSV Coat Protein Spanning The Potential Insertion Site With Introduced Base Changes and New Restriction Sites: (sequence starts at nt 3954)

I A A A N S S I N I A S V G T L Y
ATAGCCGCAGCTAACAGCTCCATAAACATAGCTAGTGTGGGTACTCTTTAT
↓ ↓
CTGCAG GGTACC
*Pst*I *Kpn*I

FIG. 10(b)

Series of Sequences to be Inserted Between the Restriction Sites to Insert the MUC1(16) Epitope at Various Locations.

G V T S A P D T R P A P G S T A
GGTGTACTTCTGCTCCTGATACTAGACCTGCTCCTGGTTCTACTGCT
CCACAATGAAGACGACCACTATGATCTGGACGAGGACCAAGATGACGA

←

GCTAACAGC
ACGTCGATTGTCG

TCCATAAACATAGCTAGTGTGGGTAC
AGGTATTTGTATCGATCACACC

GCTAACAGCTCC
ACGTCGATTGTCGAGG

ATAAACATAGCTAGTGTGGGTAC
TATTTGTATCGATCACACC

GCTAACAGCTCCATA
ACGTCGATTGTCGAGGTAT

AACATAGCTAGTGTGGGTAC
TTGTATCGATCACACC

GCTAACAGCTCCATAAAC
ACGTCGATTGTCGAGGTATTTG

ATAGCTAGTGTGGGTAC
TATCGATCACACC

GCTAACAGCTCCATAAACATA
ACGTCGATTGTCGAGGTATTTGTAT

GCTAGTGTGGGTAC
CGATCACACC

GCTAACAGCTCCATAAACATAGCT
ACGTCGATTGTCGAGGTATTTGTATCGA

AGTGTGGGTAC
TCACACC

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FIG. 11

Lipman-Pearson alignment of RCNMV and TBSV coat protein sequences.

Lipman-Pearson Protein Alignment

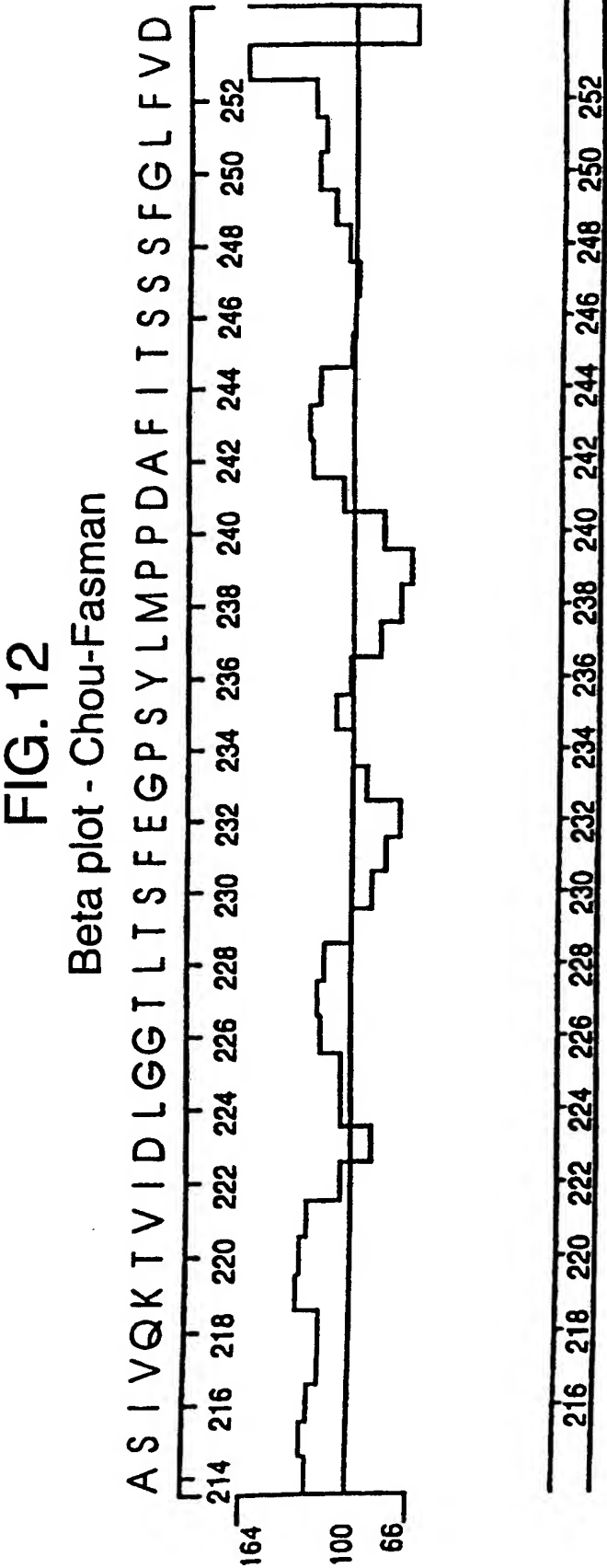
Ktuple: 2; Gap Penalty: 4; Gap Length Penalty: 12

Seq1(1>389)	Seq2(1>340)	Similarity	Gap	Gap	Consensus
tbsvts.PRO	rcnmvdia.PRO	Index	Number	Length	Length
(64>387)	(8>338)	26.9	4	7	331

```

      70      80      90     100     110     120
KKQOMINHVGGTGGAI MAPVAVTROLV GSKPKFTGRTSGSVTVTHREYLSQVNNSTGFQV
K.:Q. . . . T . . . :VA: . . . . . . . . . . : : H : : V .S. . .
KSKORSQPRNRTPTSVKTVAIPFAKTQIIKTVNPPPKPARGILHTQLVMSVVGSVQMRT
      10      20      30      40      50      60
      130     140     150     160     170     180
NGGIVGNLLQLNPLNGTLFSWLPAIASNFDOYTFNSVVLHYVPLCSTTEVGRVAIYFDKD
N.G .: .: LNP N :LF: L: A:N:D Y : .: .: L:YVPL .: : GRVA: .D D
NNGKSNQRFRLNPSNPALFPTLAYEAANYDMYRLKKLTLRYVPLVTQNSGRVAMIWDPD
      70      80      90     100     110     120
      190     200     210     220     230     240
SEDEPEADRVELANYSVLKETAPWAEAMLRVPTDKIKRFCCDSSTSDHKLIDLGLGIAT
S:D:.P..R E:.:YS .TA . . . L :P:D: RF .D::T D:KL:D:GOL .:T
SQDSAPOSROEISAYSRSVSTAVYEKCSLTIPADNQWRFVADNTTVDRKLVDFGQLLFVT
      130     140     150     160     170     180
      250     260     270     280     290     300
YGGAGTNAVGDIFISYSVTLYFPOPTNTLLSTRRLDLAALVTASGPGYLLVSR---TAT
.:G:.. .GDIF:..V.: POPT:..: . :DL:G:L.: .GP:YL: : T:
HSGSDGIETGDIFLDCEVEFKGPOPTASIVQKTVIDLGGTLTSFEGPSYLMPPDAFITSS
      190     200     210     220     230     240
      310     320     330     340     350
VLTMTFRATGTFFVISGTYRCLTATTLGLAG--GVNVNSITVVDNIG-TDSAFFINCTVSN
.: .:GT:..: . C T:..:G .: .: .: .: .:S F:..V :
SFGLFVDVAGTYLLTLVVTCTTGTSVTGNGSTLVGDGRAAYGSSNYIASIVFTSSGVLS
      250     260     270     280     290     300
      360     370     380
LPSVVTFT-STGITSATVHCVRATRONOVSL
.: V F: S:G:..: .: R .: N. L
TTPSVQFSGSSGVSRVQMNICRCKQGNTFIL
      310     320     330

```



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FIG. 14(a)

Sequence of RCNMV Coat Protein Spanning The Potential Insertion Site With Introduced Base Changes and New Restriction Sites: (sequence starts at nt 3070)

S I V Q K T V I D L G G T L T S F
 AGCATCGTACAGAAACTGTAATTGATCTCGGTGGGACACTCACTTCTTTC
 ↓ ↓ ↓ ↓
 GTGCAC GTTAAC
*Apa*Li *Hpa*I

FIG. 14(b)

Series of Sequences to be Inserted Between the Restriction Sites to Insert the MUC1(16) Epitope at Various Locations

G V T S A P D T R P A P G S T A
 GGTGTTACTTCTGCTCCTGATACTAGACCTGCTCCTGGTTCTACTGCT
 CCACAATGAAGACGACCACTATGATCTGGACGAGGACCAAGATGACGA

↙ ↘

GAAAACTGTA ACGTCTTTTGACAT	ATTGATCTCGGTGGGACGTT TAACTAGAGCCACCCTGCAA
GAAAACTGTAATT ACGTCTTTTGACATTAA	GATCTCGGTGGGACGTT CTAGAGCCACCCTGCAA
GAAAACTGTAATTGAT ACGTCTTTTGACATTAATA	CTCGGTGGGACGTT GAGCCACCCTGCAA
GAAAACTGTAATTGATCTC ACGTCTTTTGACATTAAGTAGAG	GGTGGGACGTT CCACCCTGCAA
GAAAACTGTAATTGATCTCGGT ACGTCTTTTGACATTAAGTAGAGCCA	GGGACGTT CCCTGCAA
GAAAACTGTAATTGATCTCGGTGGG ACGTCTTTTGACATTAAGTAGAGCCACCC	ACGTT TGCAA

SEQUENCE LISTING

<110> The Dow Chemical Company
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 <213> Artificial Sequence
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 Val Thr Ser Ala
 20
 35 <210> 2
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 <212> PRT
 <213> Artificial Sequence
 40 <220>
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 45 Pro Asp Thr Arg Pro
 1 5
 <210> 3
 50 <211> 5
 <212> PRT
 <213> Artificial Sequence
 <220>
 55 <223> MUC1 5mer
 <400> 3
 Ala Pro Asp Thr Arg
 1 5

-2-

5 <210> 4
 <211> 8
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 <213> Artificial Sequence

10 <220>
 <223> MUC1 8mer

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 1 5

15

 <210> 5
 <211> 8
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 <220>
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 1 5

30 <210> 6
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 <213> Artificial Sequence

35 <220>
 <223> MUC1(16)

 <400> 6
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 40 1 5 10 15

 <210> 7
 <211> 23
 45 <212> PRT
 <213> Artificial Sequence

 <220>
 <223> MUC1(23)

50 <400> 7
 Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His Gly
 1 5 10 15

55 Val Thr Ser Ala Pro Asp Thr
 20

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 00/03500

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N15/40 C12N15/62 C12N7/01 C07K14/08
A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, EMBASE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 37095 A (DANA FARBER CANCER INST INC ;THERION BIOLOG CORP (US); GRITZ LINDA) 27 August 1998 (1998-08-27) see the whole document ---	1-22
Y	WO 96 02649 A (AXIS GENETICS LTD ;LOMONOSSOFF GEORGE PETER (GB)) 1 February 1996 (1996-02-01) see the whole document ---	1-22
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

23 January 2001

Date of mailing of the international search report

30/01/2001

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Authorized officer

Celler, J

INTERNATIONAL SEARCH REPORT

Int. J. Application No

PCT/GB 00/03500

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>BALLOUL J - M ET AL: "RECOMBINANT MUC 1 VASSINIA VIRUS: A POTENTIAL VECTOR FOR IMMUNOTHERAPY OF BREAST CANCER" CELLULAR AND MOLECULAR BIOLOGY,US,TARRYTOWN, NY, vol. 40, no. 1, 1994, pages 49-59, XP000561067 ISSN: 0145-5680 see the whole document</p>	1-22
Y	<p>AKAGI J ET AL: "Therapeutic antitumor response after immunization with an admixture of recombinant vaccinia viruses expressing a modified MUC1 gene and the murine T-cell costimulatory molecule B7" JOURNAL OF IMMUNOTHERAPY,US,RAVEN PRESS, NEW YORK, vol. 1, no. 20, 1997, pages 38-47, XP002075464 ISSN: 1053-8550 see the whole document see page 42, figure 1 regarding claim 20</p>	1-22
A	<p>WO 98 56933 A (JOHN INNES CENTRE ;LOMONOSSOFF GEORGE PETER (GB); TAYLOR KATHRYN M) 17 December 1998 (1998-12-17) see the whole document</p>	1-22
A	<p>GRAHAM R A ET AL: "The polymorphic epithelial mucin: potential as an immunogen for a cancer vaccine" CANCER IMMUNOLOGY AND IMMUNOTHERAPY,DE,BERLIN, vol. 2, no. 42, 1996, pages 71-80, XP002075466 ISSN: 0340-7004 see the whole document</p>	1-22
Y	<p>WO 98 50527 A (LONGENECKER B MICHAEL ;BIOMIRA INC (CA); KRANTZ MARK J (CA); AGRAW) 12 November 1998 (1998-11-12) see whole document page 49, claim 14 regarding present Seq ID No 6</p>	1-22

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